2005

Aging affects stretch-induced p70S6k and 4E-BP1 phosphorylation in fast- and slow-twitch muscle

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AGING AFFECTS STRETCH-INDUCED $p70^{S6k}$ AND 4E-BP1 PHOSPHORYLATION IN FAST- AND SLOW-TWITCH MUSCLE

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Graduate College

Department of Biological Science

Thesis for Master of Science (M.S.)

in Biological Sciences

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04-18-05

ABSTRACT

In the present investigation we compare the expression, basal activation and the ability of muscle stretch to activate the $p70^{S6k}$ pathway in the fast-twitch extensor digitorum longus (EDL) and slow-twitch soleus of adult (6 mo. old), aged (30 mo. old) and very aged (36 mo. old) Fischer 344 x Brown Norway rats. Immunoblotting demonstrated that the tissue content of mTOR, $p70^{S6k}$, 4E-BP1 and GSK-3β decreased in the EDL and soleus in aged rats, while SHP-2 increased in the EDL and decreased in the soleus when compared to adult rats. Basal phosphorylation of 4E-BP1 increased in both the muscles; however, basal phosphorylation of mTOR increased in EDL while it was decreased in the very aged soleus. Conversely, the basal phosphorylation of GSK-3β and SHP-2 decreased in both the muscles in aged rats. Twenty percent stretch of the EDL muscle increased phosphorylation of 4E-BP1, mTOR and GSK-3ß in younger rats and decreased the phosphorylation of these molecules in the older rats. The "mTOR"- mediated phosphorylation of $p70^{S6k}$ (THR 389) decreased in all the three age groups in the EDL, while "ERK1/2"- mediated phosphorylation of $p70^{S6k}$ (THR421/SER424) increased in all the three age groups in the EDL, but increased only in the aged in soleus. Conversely, SHP-2 phosphorylation decreased in the aged soleus. Taken together, these data imply that aging affects the expression, basal phosphorylation and mechanical regulation of different signaling molecules in the rat EDL and soleus.

Key Words: EDL and Soleus; mTOR, $p70^{S6k}$, GSK-3β, 4E-BP1, SHP-2, mechanotransduction, aging

DEDICATION

The author wishes to dedicate the work to her husband, parents and kids.

ACKNOWLEDGMENTS

- Dr. Eric Blough
- Dr. Nicki Locassio and Dr. David Mallory
- Muscle physiology lab
- National Institute of Aging Grant AG-20370 and by NSF EPSCOR to Marshall University.

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LIST OF SYMBOLS

AAALAC	Association for assessment and accreditation of laboratory animal care
CSA	Cross sectional area
EDL	Extensor digitorum longus
eIF-4	Eukaryotic initiation factor -4
F344XBN	Fisher 344 Brown Norway hybrid
GSK-3ß	Glycogen synthase kinase 3 beta
IGF-1	Insulin like growth factor-1
IOD	Integrated optical densities
KRB	Krebs-Ringers Buffer solution
MAPK	Mitogen activated protein kinase
mTOR	Mammalian target of rapamycin
NF-AT	Nuclear factor activating T cells
$p70^{S6K}$	p70 ribosomal s6 kinase
ROS	Reactive oxygen species
SAPK	Stress activated protein kinase
SHP-2	Src homology protein-2
Sol	Soleus
4E-BP1	Eukaryotic initiation factor 4E binding protein-1
5'TOP	5’ terminal oligopyrimidine peptide

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CHAPTER 1

INTRODUCTION

Sarcopenia is the loss of muscle mass and strength that occurs in all adults with normal aging [1, 2]. It is estimated that sarcopenia appears to begin in the fourth decade of life and accelerates during seventh decade of life [2]. The etiology of sarcopenia is unclear, but several factors including physical inactivity, motor-unit remodeling, decreased hormone levels, and decreased protein synthesis have been implicated in development of this disorder [3]. Age-associated muscle loss and dysfunction is believed to play a major role in the pathogenesis of frailty and functional impairment that occurs with old age [4, 5]. The estimated direct healthcare cost attributable to sarcopenia in the United States in 2000 was $18.5 billion and as the number of older Americans is increasing, the economic costs of sarcopenia will escalate [6]. It is thought that the effects of sarcopenia may be delayed or largely prevented if muscle tissue could be induced to hypertrophy [5, 7, 8]. In adult men and women, it is well accepted that progressive resistance training acts to increase muscle fiber cross sectional area and mass and that these adaptations are diminished with increasing age [8]. Previous studies using the Fisher 344 Brown Norway (F344XBN) rat strain have demonstrated that aged F344XBN hybrid animals exhibit a large degree of age-associated muscle atrophy and a diminished ability to exhibit muscle hypertrophy in response to a strong hypertrophic stimulus [7, 9]. The magnitude of these age related deficiencies seem to be unique to this particular strain of rats and imply that this model may be highly suited to the mechanistic study of aged human muscle adaptation to increased

mechanical loading [9-11]. The cellular and molecular mechanism(s) which underlie the diminished ability of aged muscle to exhibit hypertrophy are not well understood.

Increased protein synthesis, an obligatory step in cellular growth and hypertrophy, is thought to be regulated by changes in translational efficiency [12-14]. The factors controlling this stage of protein synthesis are not well understood, however, it has been well established that the rate of translational initiation appears to be the rate limiting step in determining the overall rate protein synthesis [15]. In the last few years, two factors, the 70-kDa ribosomal S6 kinase ($p70^{S6k}$) and eukaryotic initiation factor 4E binding protein-1 (4E-BP1) have been identified as critical regulators in the control of initiation [14, 16-27]. Mammalian target of rapamycin (mTOR) is a direct target for protein kinase B (PKB)/AKT and this subsequently leads to increased phosphorylation of 4EBP1 and the activation of $p70^{S6k}$ [16, 27, 28]. 4E-BP1 is a low molecular weight repressor of translation initiation. When 4E-BP1 is in a dephosphorylated state, it is tightly bound to eukaryotic initiation factor (eIF)-4E [14]. 4E-BP1 binds to and regulates the availability of eIF4E, which is thought to be one of the rate limiting factors for translational initiation [14, 29]. During stimulation, as 4E-BP1 is phosphorylated, eIF-4E breaks away and initiates translation, resulting in protein synthesis [14], [30]. The $p70^{S6k}$ is a kinase whose activation is dependent upon phosphorylation. $p70^{S6k}$ when activated, acts to phosphorylate the 40S ribosomal protein, S6, at multiple sites, this phosphorylation in turn, causes an increase in the translation of mRNAs, and ultimately, an increase in protein synthesis [30]. This information is important: members of this class transcript are involved in cell cycle progression and the translation machinery (e.g. elongation factors, ribosomal proteins) [30]. Illustrating this fact, is the finding that blockade of $p70^{S6k}$ activity results in significant inhibition of protein synthesis in multiple cell systems [31, 32]. Besides the positive regulators of protein

synthesis, the activation of glycogen synthase kinase (GSK-3β) is thought to negatively regulate muscle hypertrophy [33, 34] with several studies suggesting that hypertrophic stimuli inhibit the activity of this protein by inducing its phosphorylation [35]. The mechanism by which GSK-3β influences muscle growth is not certain, but this signaling protein has been implicated in the regulation of calcineurin substrate NFAT and the protein translation initiation factor e1F2B [33]. SHP-2 is a protein tyrosine phosphatase prominently expressed in skeletal muscle and may function in a role similar to GSK-3ß, although this is controversial. Indeed SHP-2 has found to be positively and negatively regulate muscle growth [36]. This protein is reported to be essential in signaling the mitogenic effect of angiotensin II while also mediating the mitogenic effects of insulin-like growth factor 1 (IGF-1) in cultured cell lines [37]. How mTOR, $p70^{S6k}$, 4E-BP1, GSK-3ß and SHP-2 may be regulated with aging in skeletal muscle is not well understood.

Although resistance training and hypertrophy experiments have proven to be effective in younger individuals in building the muscle mass, their role in older individuals has been equivocal. Altered ability of the older muscles to adapt to contractile stimulus could be associated with altered intracellular signaling regulating protein synthesis. The understanding of this process, if present, may help us to better pharmacologically treat sarcopenia.

PURPOSE

Our long term goal is identify the cellular and molecular mechanisms in skeletal muscle responsible for sarcopenia. The purpose of this investigation was two folded. Firstly, we examined how aging may alter the expression and basal phosphorylation mTOR, $p70^{S6k}$, 4E-BP1, GSK-3ß and SHP-2. These molecules were chosen on the basis of previous data indicating that they are required for protein synthesis. Analysis of the data in the present study had allowed us to better understand the effects on aging on protein synthesis. Based on the information from the previous studies, that hypertrophic stimulus does not induce hypertrophy, we chose to apply hypertrophic stimulus to the muscles, which is twenty percent stretch. The second purpose was to determine the degree of stretch induced phosphorylation of mTOR, $p70^{S6k}$, 4E-BP1, GSK-3ß and SHP-2 in the EDL and soleus muscles with aging. This information had provided insight regarding how mechanical load regulates the activity of these molecules with aging. The rationale for the proposed research was that once it is known how mTOR, $p70^{S6k}$, 4E-BP1, GSK-3ß and SHP-2 are regulated with aging and load, their activation or repression may be regulated pharmacologically to prevent or treat sarcopenia.

SPECIFIC AIMS

The objective of this study was to determine how mTOR, $p70^{S6k}$, 4E-BP1, GSK-3ß and SHP-2 phosphorylation are regulated in aged-atrophic skeletal muscle with stretch. We hypothesized that mTOR, $p70^{S6k}$, 4E-BP1, GSK-3ß and SHP-2 phosphorylation with stretch will be attenuated with increasing age in the F344XBN hybrid rat strain. In the present study, we compared the extent and time course of mTOR, $p70^{S6k}$, 4E-BP1, GSK-3ß and SHP-2 phosphorylation after twenty percent stretch in isolated fast- and slow-twitch skeletal muscles from different age groups of F344XBN.

Specific Aim 1:

To determine the basal tissue content of mTOR, $p70^{S6k}$, 4E-BP1, GSK-3ß and SHP-2 in EDL and soleus with aging in F344XBN hybrid rats To examine this possibility, the EDL (fast twitch) and soleus (slow twitch) muscles of rats (6-, 30-, and 36-months) of age were harvested, homogenized and subjected to immunoblotting analysis. Alterations in the expression of mTOR, $p70^{S6k}$, 4E-BP1, GSK-3ß and SHP-2 in EDL and soleus in the three age groups were evaluated by densitometry and statistical methods.

Hypothesis:

We hypothesized that age associated decrease in muscle hypertrophy were related to decreases in the tissue content of mTOR, $p70^{S6k}$, 4E-BP1, GSK-3ß and SHP-2 in the EDL and soleus.

Specific Aim 2:

To determine the degree of stretch-induced phosphorylation of mTOR, $p70^{S6k}$, 4E-BP1, GSK-3ß and SHP-2 in EDL and soleus with aging in F344XBN hybrid rats.

EDL (fast twitch) and soleus (slow twitch) muscles of rats (6-, 30-, and 36-months) of age muscles were harvested and muscles were subjected to twenty percent stretch for either 5 or 15 minutes in oxygenated (95% O_2, 5% CO_2) Krebs-Ringer bicarbonate buffer maintained at 20ºC. Phosphorylation levels were evaluated by immunoblotting, phosphospecific antibodies and densitometry.

Hypothesis:

We hypothesized that age associated decreases in muscle hypertrophy are related to a decreased ability of aged muscles to phosphorylate mTOR, $p70^{S6k}$, 4E-BP1, GSK-3ß and SHP-2 in response to increased mechanical load.

CHAPTER 2

REVIEW OF THE LITERATURE

INTRODUCTION

The following chapter presents a review of the pertinent literature concerning the present study. Specifically, the following areas will be addressed: 1) age associated alterations in skeletal muscle and 2) the regulation of protein synthesis.

Age associated alterations in skeletal muscle

Strength in humans has been reported to decline by the fourth [38] or only after the eighth decade [39]. Losses in maximal voluntary contractions (MVC) [40, 41] have ranged from 26-38% in the quadriceps to 10-47% in the triceps surae [39, 42]. A great deal of research on aging has been performed on the aged rats because of the difficulties associated with aging studies in human e.g. ethical, cross sectional design and inability to control life time activity pattern of the subjects. As with human beings, aging in animals also appears to affect muscle function. For example, decreases in the maximal *in vitro* isometric twitch and tetanic forces of 20-35% have been reported for muscles of 26 month old compared with adult mice [43-45] and rats, [46-49] with similar deficits for both slow and fast muscles [43, 44]. The loss of muscular strength with age may be related in part to a loss of muscle mass. Young *et al.* (1984, 1985), [50, 51] used ultrasonography and found 25-35% reductions in the cross sectional area of the quadriceps muscle in men and women over the age of 65 years when compared to young. The computed tomography scanning technique has shown similar age-related reductions in cross sectional area

of the psoas major and sacrospinalis muscles, [52] the quadriceps muscle, [53] the brachial biceps and triceps muscles [54, 55] and the plantarflexors [54, 55] of men over the age of 65.

Similar to the aging human, research utilizing aged rats found similar declines in muscle mass [56-61]. The extent of the decline has been found to be variable and dependent upon the function of the muscle [3, 62-64]. Postural muscles appear to be affected more than non-postural muscles in rats [3, 62, 65]. Reduction in muscle mass may be due to decreases in muscle fiber area. Within the limitations of cross sectional studies, type I fiber areas appear to undergo little change until the age of 60-70 years, [66, 67] but changes in type II fibers are less clear. Type II fiber area has been reported to decline by 40% in subjects aged 60-65 years compared to subjects aged 20-29 years [67, 68]. However, Aniansson and colleagues, (1980) [69] found only minor area reductions until the seventh decade in humans. In rodents, several investigators have noted a similar, preferential reduction in type II fiber cross sectional area (CSA) and little or no decline in type I fiber CSA [70, 71] but others have disputed this, [3, 72, 73] ~30% reduction in the average CSA of type II fibers of the plantaris in aged 28-30 month old Long Evans rats compared to 9-10 month old animals. The important conclusion drawn from the above work is that age associated changes in fiber cross sectional area (CSA), like muscle mass, may be manifested differently in various muscle fiber types and also depend on whether the muscles in question are weight bearing or non-weight bearing [3].

Skeletal muscle exhibits a great deal of plasticity, which is specific to the stimulus it receives [74, 75]. Hypertrophy of muscle cells in response to increased functional demand is a well established phenomenon. It has been shown that resistance training in humans leads to muscle hypertrophy which is mainly the result of an enhanced cross-sectional area of individual muscle fibers [76, 77]. Similar adaptations have been observed in animals after ablation or

denervation of synergist muscle [77, 78]. Muscle plasticity or the ability of muscle to adapt to an altered contractile stimulus is decreased with aging in both humans and animals [4, 9, 11, 40, 58, 79]. The mechanism(s) underlying age-associated changes in muscle plasticity are unknown.

Summary

The age associated loss of muscle mass leads to a reduced force generating ability and eventually, a decreased functional capacity. Due to the problems associated with performing invasive measures in a human population along with a lack of molecular investigations, the underlying mechanisms responsible for the documented alterations are unknown.

Protein synthesis, p70S6k, and 4E-BP1

Contractile activity and mechanical overload in skeletal muscle stimulate protein synthesis, leading to enhanced fiber size and strength, and alterations in metabolic properties [80-84]. Protein synthesis is thought to be regulated by changes in translational efficiency [15]. The factors controlling translational efficiency are not well understood, however it has been well established that the rate of translational initiation appears to be the rate limiting step in determining the overall rate of protein synthesis [12, 14, 15].

Two factors, the 70-kDa ribosomal S6 kinase ($p70^{S6k}$) and eukaryotic initiation factor 4E binding protein-1 (4E-BP1) have been identified as critical regulators in the control of initiation and increases in muscle cell size [14, 16, 21, 27, 85-87]. Baar and Esser 1999 demonstrated an increase in the rate of initiation associated with increase in the phosphorylation of p70S6k in the tibilais anterior and EDL muscles following high-resistance lengthening contractions. Similarly, $p70^{S6k}$ and 4E-BP1 have been found to be activated during hypertrophy in the *in vitro* hypertrophy of C2C12 muscle cells [27]. The activation of $p70^{S6k}$ is thought to result in the selective translation of a set of growth-related mRNA containing a 5' terminal polypyrimidine tract (5'TOP) [32, 88-92]. This finding is important: the 5'TOP motif is found in the 5' untranslated region of elongation factors (eEF; e.g., eEF-1a and eEF-2) and ribosomal proteins (e.g., S3, S14, and S24) [32, 89, 91, 93].The immediate upstream regulator of $p70^{S6k}$ and 4E-BP1 is thought to be the mammalian target of rapamycin (mTOR) [13, 94-103]. The mTOR belongs to the PIKK family of novel Ser/Thr kinases and functions as a key regulator of cell growth and cell cycle progression [104]. Research regarding the function of mTOR in skeletal muscle is not overly abundant. Parkington, *et al.* 2004 reported increases in phosphorylation of mTOR, ERK1/2, and $p70^{S6K}$ in skeletal muscle after a single bout of *in situ* muscle contractile activity

suggesting that the activation of these factors is inter-related [94, 105]. In addition to mTOR and ERK 1/2, the kinases Akt/PKB and PDK1, PKCζ have also been implicated in activation of p70S6k *in vitro* [16, 27, 106, 107]. Genetic studies in Drosophila where the Akt gene has been activated in a constitutive fashion support the participation of Akt/PKB in the activation of mTOR [108, 109] and these findings have been substantiated in cell culture studies using muscle cell lines [16, 27]. Whether p70S6k is regulated by a similar mechanism *in vivo* is unclear.

4E-BP1 is a translation inhibitor that is phosphorylated and inactivated in response to the growth signals [110]. Previous studies have suggested that a major target of p42mapk / p44mapk (ERK1/2) activation following insulin stimulation of fat cells is 4E-BP1 [23, 111-113]. Phosphorylation of 4E-BP1 disrupts its interaction with protein synthesis initiation factor eIF-4E, liberating eIF-4E to interact with the p220 subunit of the mRNA cap binding protein complex [85, 114]. Similar to p70S6k, *in vitro* investigations using inhibitors have implicated the mTOR as an upstream regulator of 4E-BP1 [115]. How 4E-BP1 is regulated in differentiated skeletal muscle has not been fully elucidated.

Negative regulators of protein synthesis

Glycogen synthase kinase-3 (GSK-3) [116] was originally discovered as a protein kinase that phosphorylates and inactivates glycogen synthase [117]. Molecular cloning from a rat brain library revealed two GSK-3 isoforms termed α and ß which are 98% identical in their kinase domains [118]. GSK-3ß is thought to exert an anti-hypertrophic effect by affecting nuclear/cytoplasmic partitioning of the endogenous nuclear factor activating T (NF-ATs), [33, 119, 120] c-jun, [121-123] the eukaryotic initiation factor (eIF-2B) [124] , tau [125]or the transcriptional activator ß –catenin [33, 126]. GSK-3ß is phosphorylated (inactivated) by

phosphorylation of serine residue 9 in response to growth factors, especially insulin and IGF-1 [16, 27, 33, 127]. How GSK-3ß is regulated with aging in the skeletal muscle is not known.

SHP-2 is a widely abundant tyrosine phosphatase and very little is known and understood about the mechanism of action of SHP-2. SHP2 was recently found to downregulate PI3K activation by dephosphorylating Gab1 [128]. Accumulating evidence implicates SHP-2 as a positive regulator of ERK activity downstream from receptor-tyrosine kinases [129, 130]. SHP-2 activity is critical for the regulation of downstream events activated by growth factors [131, 132] however, it is unclear how SHP-2 is regulated in skeletal muscle with aging.

Summary

It has been well established that the rate of translational initiation appears to be the rate limiting step in determining the overall rate of protein synthesis. Previous *in vitro* studies have suggested that activation of p70S6k and 4E-BP1 might serve as a crucial regulator of muscle fiber growth and mTOR is thought to function as the upstream activator of p70S6k and 4E-BP1; however *in vivo* substantiation of these data has been equivocal. Smooth muscle and cardiac muscle *in vitro* hypertrophy models have demonstrated GSK-3ß and SHP-2 as potential negative regulators of protein synthesis. How GSK-3ß and SHP-2 may be regulated *in vivo* and with aging in skeletal muscle has not been investigated.

CHAPTER 3

AGING AFFECTS STRETCH-INDUCED $p70^{S6k}$ AND 4E-BP1 PHOSPHORYLATION IN FAST- AND SLOW-TWITCH MUSCLE

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ABSTRACT

In the present investigation we compare the expression, basal activation and the ability of muscle stretch to activate the $p70^{S6k}$ pathway in the fast-twitch extensor digitorum longus (EDL) and slow-twitch soleus of adult (6 mo. old), aged (30 mo. old) and very aged (36 mo. old) Fischer 344 x Brown Norway rats. Immunoblotting demonstrated that the tissue content of mTOR, $p70^{S6k}$, 4E-BP1 and GSK-3β decreased in the EDL and soleus in aged rats, while SHP-2 increased in the EDL and decreased in the soleus when compared to adult rats. Basal phosphorylation of 4E-BP1 increased in both the muscles, however, basal phosphorylation of mTOR increased in EDL while it was decreased in the very aged soleus. Conversely, the basal phosphorylation of GSK-3β and SHP-2 decreased in both the muscles in aged rats. Twenty percent stretch of the EDL muscle increased phosphorylation of 4E-BP1, mTOR and GSK-3ß in younger rats and decreased the phosphorylation of these molecules in the older rats. The "mTOR"- mediated phosphorylation of $p70^{S6k}$ (THR 389) decreased in all the three age groups in the EDL, while "ERK1/2"- mediated phosphorylation of $p70^{S6k}$ (THR421/SER424) increased in all the three age groups in the EDL, but increased only in the aged in soleus. Conversely,

SHP-2 phosphorylation decreased in the aged soleus. Taken together, these data implicate that aging affects the expression, basal phosphorylation and mechanical regulation of different signaling molecules in the rat EDL and soleus.

INTRODUCTION

Sarcopenia is the loss of muscle mass and strength that occurs in all adults with normal aging [1, 2]. Age-associated muscle loss and dysfunction is believed to play a major role in the pathogenesis of frailty and functional impairment that occurs with old age [4, 5]. It is thought that the effects of sarcopenia may be delayed or largely prevented if muscle tissue could be induced to hypertrophy [5, 7, 8]. In adult men and women it is well accepted that progressive resistance training acts to increase muscle fiber cross sectional area (CSA) and mass and that these adaptations are diminished with increasing age [8]. Previous studies by our laboratory and others using the Fisher 344 Brown Norway F1 hybrid (F1 hybrid) rat strain have demonstrated that aged F1 hybrid animals exhibit a large degree of age-associated muscle atrophy and a diminished ability to exhibit muscle hypertrophy in response to a strong hypertrophic stimulus [3, 9-11, 59, 95]. These finding suggest that similar to most human studies, muscle adaptability in these animals decreases with age. The magnitude of these age related deficiencies seem to be unique to this particular strain of rats and imply that this model may be highly suited to the mechanistic study of aged human muscle adaptation to increased mechanical loading [9, 11, 59, 133]. The cellular and molecular mechanism(s) which underlie the diminished ability of aged muscle to exhibit hypertrophy are not well understood.

Increased protein synthesis, an obligatory step in cellular growth and hypertrophy, is thought to be regulated by changes in translational efficiency [12-14]. The factors controlling this stage of protein synthesis are not well understood, however it has been well established that the rate of translational initiation appears to be the rate limiting step in determining the overall rate protein synthesis [12, 14, 15]. In the last few years, two factors, the 70-kDa ribosomal S6 kinase (p70S6k) and eukaryotic initiation factor 4E binding protein-1 (4E-BP1) have been

identified as critical regulators in the control of initiation [14, 16-27]. 4E-BP1 is a low molecular weight repressor of translation initiation. When 4E-BP1 is in a dephosphorylated state, it is tightly bound to eukaryotic initiation factor (eIF)-4E [14]. 4E-BP1 binds to and regulates the availability of eIF4E, which is thought to be one of the rate limiting factors for translational initiation [14, 29]. During stimulation, as 4E-BP1 is phosphorylated, eIF-4E breaks away and initiates translation, resulting in protein synthesis [16]. The $p70^{S6k}$ is a kinase whose activation is dependent upon phosphorylation [134]. $p70^{S6k}$ when activated, acts to phosphorylate the 40S ribosomal protein, S6, at multiple sites, this phosphorylation in turn, causes an increase in the translation of mRNAs, and ultimately, an increase in protein synthesis [26]. The $p70^{S6k}$ is thought to play a critical role in the translation of a class of mRNA transcripts that contain an oligopyrimidine tract at their transcriptional start site [26]. This fact is important because members of this class transcription factors are involved in cell cycle progression and the translation machinery (e.g. elongation factors, ribosomal proteins) [17, 30]. Illustrating this fact is the finding that blockade of $p70^{S6k}$ activity results in significant inhibition of protein synthesis in multiple cell systems [25, 31, 32, 135]. Recent investigations using adult animals have suggested that the degree of $p70^{S6k}$ activation with increased contractile demand is related to phenotypic adaptation [9, 95]. For example, Baar and Esser, (1999) demonstrated a significant correlation ($r = 0.998$) between increases in $p70^{S6k}$ phosphorylation and percent increase in muscle mass with resistance training [99]. Moreover, two elegant studies, using multiple *in vitro* [136, 137] and *in vivo* models [99, 138] of muscle plasticity and genetic intervention demonstrated that activation of $p70^{S6k}$ and 4E-BP1 are requisitely involved in regulating skeletal muscle fiber size [138]. In addition to contractile stimuli, muscle stretch has been established to activate $p70^{S6k}$. Torgan and others (2000) demonstrated using muscle myocytes cultured on

deformable membranes that increased stretch is able to activate $p70^{S6k}$ in a time and stretch dependent manner [136, 139]. In a similar fashion Hornberger *et al.*, found that intermittent stretch of the mouse EDL is able to induce p70S6k phosphorylation [140] and in a follow up study, this same group showed that aging in mice does not alter the mechanosensitivity of the $p70^{S6k}$ signaling pathway [141]. Of interest is the finding that the EDL muscles from the CB6F1 aged mice used in the Hornberger *et al.*, study failed to exhibit decreases in whole muscle mass with aging. This absence of whole muscle atrophy is clearly different from what we and others have previously observed with aging in the Fischer344 x Brown Norway hybrid rat strain and humans [9-11]. Such differences may be important since muscle atrophy has been suggested to alter the expression of signaling proteins involved in the regulation of muscle adaptation [142-144].

The objective of this study was to determine how $p70^{S6k}$ and 4E-BP1 phosphorylation is regulated in aged - atrophic skeletal muscle with stretch. We hypothesized that $p70^{S6k}$ and 4E-BP1 phosphorylation with stretch will be attenuated with increasing age in the Fisher 344xBrown Norway F1 hybrid rat strain. Because different muscle types exhibit and undergo altered degrees of muscle growth to loading stimuli and dissimilar amounts of age-associated dysfunction and atrophy with aging, it is also unclear how $p70^{S6k}$ protein content or activity might be regulated in different muscle types soleus and EDL with aging. In the present study, we compared the extent and time course of $p70^{S6k}$ and 4E-BP1 phosphorylation after muscle stretch in isolated fast- and slow-twitch skeletal muscles from different age groups of F1 hybrid rats. The results indicate significant muscle type specific alterations in $p70^{S6k}$ and 4E-BP1 expression and stretch-induced activation with aging.

MATERIALS AND METHODS

Animals

All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals as approved by the Council of the American Physiological Society and the Animal Use Review Board of The Marshall University. All procedures were conducted in strict accordance with Public Health Service animal welfare policy. Adult (6 months), aged (30 months) and very aged (36 months) male F1 rats were obtained from the National Institute on Aging.

Rats were barrier housed two per cage in an AAALAC approved vivarium. Housing conditions consisted of a 12H: 12H dark-light cycle and temperature was maintained at 22 ± 2 °C. Animals were provided food and water ad libitum. Rats were allowed to recover from shipment for at least two weeks before experimentation began, and during this time the animals were carefully observed and weighed weekly. None of the older animals exhibited signs of failure to thrive, such as precipitous weight loss, disinterest in the environment, or unexpected gait alterations.

Materials

Anti- $p70^{S6k}$ (1:500 dilution), mTOR (1:1000 dilution), GSK-3β (1:1000 dilution), SHP-2 (1:1000 dilution), 4E-BP1(1:1000 dilution), Thr^{389} phosphorylated $p70^{S6k}$ (1:500 dilution), ^{Ser421Thr429} phosphorylated $p70^{S6k}$ (1:500 dilution), ^{ser2448} phosphorylated mTOR (1:1000 dilution), ^{Ser9} phosphorylated GSK-3β (1:1000 dilution), phosphorylated SHP-2 (1:1000 dilution), phosphorylated 4E-BP1(1:500 dilution) mouse IgG, and Rabbit IgG antibodies were purchased from Cell Signaling Technology (Beverly, MA). Enhanced chemiluminescence (ECL) western

blotting detection reagent was from Amersham Biosciences (Piscataway, NJ). Restore western blot stripping buffer was obtained from Pierce (Rockford, IL) and 3T3 cell lysates were from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were purchased from Sigma (St. Louis, MO).

Muscle incubation

Rats were anesthetized with a ketamine-xylazine (4:1) cocktail (50 mg/kg ip) and supplemented as necessary for reflexive response. In a sterile environment, the dorsal surface of the hind limb was shaved, cleaned, and the soleus and extensor digitorum longus (EDL) muscles were isolated using blunt dissection. Calipers were used to determine the in situ (L_o) resting muscle length of Sol and EDL at 90º ankle flexion. Once removed from the animal, suture (2.0) was tied to the proximal and distal tendons and the muscles mounted in a custom designed incubation chamber at L_o. Dissection and mounting procedures were performed rapidly and with care to prevent stretching or tearing of the muscle. After 15 minutes of equilibration, muscles were subjected to 20% stretch for either 5 or 15 minutes. All muscle incubations were performed in oxygenated (95% O_2, 5% CO_2) Krebs-Ringer bicarbonate buffer maintained at 20ºC. After stretch, muscles were weighed and flash frozen in liquid N_2.

Western blotting

Soleus and EDL muscles were homogenized on ice for 2 X 30 s in T-PER (2mL/100mg tissue weight) supplemented with 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF). After homogenization and centrifugation (9000 X g X 60 min. at 4ºC), the supernatant was separated from the pellet and stored in aliquots at -80ºC until use. Protein concentrations of the

supernatant were determined in triplicate using BSA as a standard and the Bradford method (Pierce, Rockford, IL). Samples were diluted to a concentration of 3mg/ml in SDS-loading buffer and after boiling for 5 minutes, 60 μg of total protein for each age or time point were separated on 7.5% SDS-PAGE gels. Western blot transfer of protein onto nitrocellulose membranes was performed using standard conditions. To verify transfer of proteins and equal loading of lanes the membranes were stained with Ponceau S. For immunodetection, membranes were blocked in 5% Milk TBST for 1 hour at room temperature and then incubated with the appropriate primary antibody overnight. After washing in TBST, the membranes were exposed to horseradish peroxidase-labeled IgG secondary antibody for 1 hour at room temperature. Protein bands were visualized with ECL (Amersham Biosciences). Exposure time was adjusted to keep the integrated optical densities (IODs) within a linear and nonsaturated range, and band signal intensity was quantified by densitometry using a flatbed scanner (Epson Pefection 3200 PHOTO) and Imaging software (AlphaEaseFC). Molecular weight markers (Cell Signaling) were used as molecular mass standards and NIH 3T3 cell lysates were included as positive controls. To allow direct comparisons between expression levels of different signaling molecules, immunoblots were stripped and reprobed with Restore western blot stripping buffer as detailed by the manufacturer (Pierce, Rockford, IL). After verifying the absence of residual HRP activity on the membrane by reaction with the ECL reagent, membranes were washed and reprobed.

Data Analysis

Results are presented as mean ± SEM. Multiple group comparisons were performed by one-way ANOVA followed by post-hoc testing where appropriate. For all comparisons, the alpha level was set at $P < 0.05$.

RESULTS

Aging effects on p70^{S6K} pathway related protein expression and phosphorylation.

To investigate whether aging affected the total amount of p70^{S6k}, mTOR, 4E-BP1, GSK-3β and SHP-2 expression in the skeletal muscle, we performed protein gel electrophoresis and immunoblotting using antibodies which recognize both the unphosphorylated and phosphorylated forms of these molecules. Immunoreactive bands of ~14.5kDa, ~70kDa, ~289kDa, ~45kDa and ~72kDa corresponded to the predicted molecular mass of the 4E-BP1, p70^{S6k}, mTOR, GSK-3β and SHP-2, respectively.

Compared to the 6-month EDL (Table 1) muscle, immunoblot analysis demonstrated significant decreases of ~27.3% and ~58.39% ($P<0.05$) and ~38.7% and ~35% ($P = <0.001$), respectively in the muscle content of p70^{S6k} and mTOR in 30- and 36-month muscles (Figure 1A). In contrast, the GSK-3β and 4E-BP1 (Figure 1B) levels were not altered in the 30-month EDL muscles however, the expression of these molecules was significantly decreased ~44.4% and ~32.55% ($P<0.05$), respectively in the 36-month EDL muscles when compared to the 6-month animals. The SHP-2 levels increased ~38% in both the 30 month and 36 month in the EDL muscle (Figure 1B). When compared to the 6-month soleus (Table 2) muscles, there were significant decreases of ~37.9% and ~68.0% ($P<0.05$) in p70^{S6k} (Figure 2A), decreases of ~23.8% and 31.5 % ($P<0.05$) in GSK-3 β (Figure 2B) and decreases of ~ 58% and ~60% ($P<0.05$), in the content of 4E-BP1 (Figure 2B) in the 30- and 36- month soleus samples, respectively. The amount of mTOR (Figure 2A) and SHP-2(Figure 2B) levels were not altered in the 30-month soleus samples, however, they were significantly decreased ~47.86% ($P = <0.001$) and ~ 44.4% ($P<0.001$), respectively in the 36 month soleus muscles.

Because p70 ^{S6k}, mTOR, 4E-BP1, GSK-3 β and SHP-2 are activated or repressed by phosphorylation [16, 17, 35, 80, 145-147], it was of interest to determine if the phosphorylated form of these proteins is altered with aging in the rat skeletal muscle. The phosphospecific antibodies used for western blot analysis recognized the proteins of appropriate size p70 ^{S6k}, 4E-BP1, mTOR, GSK-3 β and SHP-2. Similar to our previous analysis investigating how aging alters the total expression of these proteins, it appears that the basal phosphorylation status of p70 ^{S6k}, mTOR, 4E-BP1, GSK-3 β and SHP-2 are also regulated differently with increasing age. In the EDL, the basal phosphorylation of mTOR (Figure 3A), 4E-BP1 (Figure 6A) and SHP-2 (Figure 8A) did not change in the 30 month muscles when compared to samples obtained from 6 month animals. Conversely, in the 36-month old EDL muscles, the mTOR and 4E-BP1 levels were increased ~219% ($P = <0.001$) and ~80.33% ($P = 0.002$), respectively while the SHP-2 were levels decreased ~20.71% ($P = 0.041$). With respect to the EDL muscles obtained from 6-month animals, the basal phosphorylation levels of the "ERK1/2 MAPK"- dependent (Thr421/Ser424) (Figure 5A) and the "mTOR"- dependent (Thr 389) (Figure 4A) form of p70 ^{S6k} were increased ~14% at 30-month ($P = <0.001$) but were not altered in the 36-month soleus. Conversely, the basal phosphorylation of the GSK-3 β decreased ~14% in the 30 month EDL muscle samples and ~36.7% in the 36 month EDL muscle samples, respectively ($P = <0.001$) (Figure 7A). Similarly, compared to the 6 month soleus, basal phosphorylation of the mTOR (Figure 3B), SHP-2 (Figure 8B) and 4E-BP1 (Figure 6B) increased ~33.4% ($P = <0.001$) ~39% ($P = 0.002$) and ~19 % ($P = <0.001$), respectively in the 30 month soleus. In the 36 month soleus, the mTOR and SHP-2 phosphorylation levels were decreased ~44.27% ($P = <0.001$) and ~59 % ($P = 0.002$), respectively. The amount of "mTOR"- mediated (Thr 389) phosphorylation of p70 ^{S6k} (Figure 4B) did not change with age while the "ERK1/2 MAPK"- mediated

(Thr421/Ser424) (Figure 5B) decreased ~23% in the 36 month (P = <0.001) soleus. Conversely, the basal phosphorylation levels of the GSK-3 β (Figure 7B) decreased ~29% at the 30 month and ~31% in the 36 month old soleus muscles, respectively (P = <0.001).

Electrophoretic determination of p70 ^{S6k} pathway and 4E-BP1 related phosphorylation in response to mechanical loading by twenty percent stretch.

In parallel studies, the effect of twenty percent static stretch on the ability of the EDL and soleus to phosphorylate mTOR, p70 ^{S6k,} 4E-BP1, GSK-3 β and SHP-2 was determined (Table 3 and 4). In the six month EDL muscles, stretch increased the phosphorylation of the mTOR ~100.83% at 15 minutes (P = <0.001) (Figure 3A). In the 30 month EDL muscle the mTOR phosphorylation did not change with stretch, while in the 36 month EDL samples the mTOR phosphorylation with stretch decreased ~229.46% at 5 minutes and ~243.77% at 15 minutes. In the 6 month EDL, the 4E-BP1 phosphorylation with stretch increased ~69% (P=<0.001) and ~98% (P=0.002) at 5 and 15 minutes, respectively (Figure 6A). Conversely, in the 30 month EDL, the 4E-BP1 phosphorylation did not change. In the 36 month EDL muscle stretch decreased the 4E-BP1 phosphorylation ~121% (P=0.002) at 15 minutes. In the 6 month EDL muscles, the GSK3β phosphorylation was increased ~98% (P=0.002) at 5 and ~7.7% (P=0.01) at 15 minutes with stretch (Figure 7A). Conversely, the stretch induced GSK3β phosphorylation was unchanged at any time point in the 30 month animals. The GSK3β phosphorylation was decreased ~13% (P=<0.001) and ~9.1% (P=0.009) at 5 and 15 minutes**,** respectively in the 36 month EDL muscles. In the EDL with stretch, the "mTOR"- dependent p70^{S6k} (Thr389) phosphorylation (Figure 4A) was decreased ~27% (P=<0.001) and ~14.7% (P = 0.001) at 5 and 15 minutes, respectively in the 6 month. Similarly, in the 30 month EDL "mTOR"- dependent p70^{S6k}

(Thr389) phosphorylation decreased by ~22% and ~24.4 % at 5 and 15 minutes, respectively (P=<0.001). With stretch, the "mTOR"- dependent $p70^{S6k}$ (Thr389) phosphorylation in the 36 month EDL decreased ~21% (P=0.006) and ~54.9% (P=0.002) at 5 and 15 minutes, respectively. Stretch induced "ERK1/2 MAPK"- dependent $p70^{S6k}$ (Thr421/Ser424) phosphorylation (Figure 5A) in the EDL increased in the 6 month muscles ~41.6% and ~81.63% (P = 0.002) at 5 and 15 minutes, respectively. Similarly, the "ERK1/2 MAPK"- dependent $p70^{S6k}$ (Thr421/Ser424) phosphorylation increased ~52.8% at 15 minutes (P = 0.003) in the 30 month old EDL muscles. In the 36 month EDL, the stretch induced "ERK1/2 MAPK"- dependent $p70^{S6k}$ (Thr421/Ser424) phosphorylation increased ~36.7% (P=<0.001) and ~32% (P = 0.015) at 5 and 15 minutes, respectively. In contrast, the SHP-2 (Figure 8A) phosphorylation did not change with twenty percent stretch at any time in the 6-, 30- and 36- month age groups. In the 6 month soleus subjected to twenty percent stretch, phosphorylation of the mTOR (Figure 3B) did not change at 5 minutes, but decreased ~21%(P=0.002) at 15 minutes. The mTOR phosphorylation with decreased ~40.28% and ~52.6% at 5 and 15 minutes, respectively (P=0.002) in the 30 month soleus. However, in the 36 month old soleus muscles, the mTOR phosphorylation with stretch did not change at 5 minutes, but decreased ~17.24%(P=0.03) at 15 minutes. Stretch induced the "mTOR"- dependent $p70^{S6k}$ (Thr389) phosphorylation (Figure 4B) in the 6 month soleus muscles decreased ~19.44% (P=0.014) 5 minutes. The "mTOR"- dependent $p70^{S6k}$ (Thr389) phosphorylation was not altered with stretch at 5 and 15 minutes in the 30- or 36-month soleus muscles. The "ERK1/2 MAPK**"**- dependent $p70^{S6k}$ (Thr421/Ser424) phosphorylation (Figure 5B) did not change at 5 min and 15 minutes in the 6 month with stretch. However**,** the "ERK1/2 MAPK**"**- dependent $p70^{S6k}$ (Thr421/Ser424) phosphorylation increased with stretch ~29% (P=<0.001) at 15 minutes in the 30 month soleus. With stretch the "ERK1/2 MAPK"- dependent

$p70^{S6k}$ (Thr421/Ser424) phosphorylation in the soleus increased ~28% and ~ 22% at 5 and 15 minutes, respectively in the 36 month (P=<0.001). In the 6 month soleus, stretch induced phosphorylation of the 4-EBP1 (Figure 6B) increased ~17% (P=0.002) and 47% (P=<0.001) at 5 and 15 minutes, respectively. Conversely, the 4E-BP1 phosphorylation did not change with stretch in the 30 month, but decreased ~39% and ~60% at 5 and 15 minutes, respectively (P=<0.001) in the 36month soleus muscle. In the 6 month soleus, the GSK-3 β phosphorylation (Figure 7B) was decreased ~25 % at 5 minutes and ~27 % at 15 minutes, respectively (P=<0.001). However, with stretch, the GSK-3 β phosphorylation decreased ~7 % (P=0.001) at 5 minutes in the 30 month with no change at 15 minutes in soleus. Similarly, in the 36 month soleus, stretch induced the GSK-3 β phosphorylation decreased ~5.6% (P=0.002) at 5 minutes with no change at 15 minutes. SHP-2(Figure 8B) phosphorylation in the soleus did not change with twenty percent stretch at 5 and 15 minutes in 6month. Conversely, the SHP-2 phosphorylation in the 30 month soleus decreased ~22% (P=0.041) at 15 minutes. On the other hand, SHP-2 phosphorylation with stretch in the 36 month soleus increased ~10% (P=0.024) at 5 minutes with no change at 15 minutes.

DISCUSSION

The major new finding of the present study is that the muscle content and the mechanically-induced activation of $p70^{S6k}$, 4E-BP1and several of its pathway-related proteins are regulated differently with aging in the rat EDL and soleus. The $p70^{S6k}$ and GSK-3β are thought to play a key role in the regulation of protein synthesis following altered loading or increased contractile activity [16, 137]. Indeed $p70^{S6k}$ has been suggested to play a critical role in the translation of transcripts involved in the cell cycle progression and the translational machinery [148]. In contrast, GSK-3β is thought to negatively regulate muscle hypertrophy [34,35] with several studies suggesting that hypertrophic stimuli inhibit the activity of this protein by inducing its phosphorylation [33, 145]. In the dephosphorylated form, 4E-BP1 is thought to partner with eIF-4E and prevent its association with eIF-G thereby acting to inhibit protein translation [14]. SHP-2 is a protein tyrosine phosphatase whose degree of phosphorylation has been shown to be positively related to its role in inhibiting the mitogenic effects of insulin-like growth factor 1 [149,150]. On the basis of these findings and a previous study demonstrating an GSK-3β phosphorylation impairs its function decreased protein synthesis rate with aging, we expected that aging would decrease the content and degree of mTOR, $p70^{S6k}$ 4E-BP1 and GSK-3β basal phosphorylation in the soleus and EDL. Using this same line of thought, we hypothesized that aging would be also be associated with an decreased degree of GSK-3β and increased SHP-2 phosphorylation. We demonstrate in this study, that while the tissue content of mTOR, $p70^{S6k}$ (Figure 1A) and 4E-BP1, GSK-3β and SHP-2(Figure 1B) proteins in EDL and mTOR, $p70^{S6k}$ (Figure 2A) and 4E-BP1, GSK-3β and SHP-2(Figure 2B) proteins in soleus was decreased significantly ($p<0.05$) with aging; the basal phosphorylation of the mTOR dependent forms of $p70^{S6k}$ and 4E-BP1 were significantly increased in the EDL of very aged rats (Figures 4A and

6A). Conversely, the basal phosphorylation of SHP-2 and GSK-3β were decreased in very aged rats in EDL and soleus (Figures 7Aand B and 8 A and B). Taken together, these alterations imply that aged-atrophying muscles are attempting to counteract protein breakdown by inducing the phosphorylation (activation) of molecules implicated in the control of protein synthesis. These findings are consistent with previous studies examining the phosphorylation status of $p70^{S6k}$ in aging skeletal muscle [94] and in the atrophic remodeling heart [151]. Because aging is characterized by significant muscle loss in this model and these data suggest that the net flux of protein catabolic processes exceeds that of protein anabolic processes. Future experiments designed to specifically address alterations in protein breakdown will no doubt help to substantiate this speculation.

Mechanical stress has been implicated as a major factor responsible for the "functional health" and adaptation of striated muscle to overload. It is well known that muscle is very sensitive to its loading state; unloading induces atrophy while overload causes hypertrophy [8, 137]. The mechanism by which muscle transmits external forces into biochemical signals is not known. Because aging is associated with a diminished response to muscle overload, [9-11] we hypothesized that aging would diminish the ability of skeletal muscle to appropriately "sense" and "respond" (initiate intracellular signaling) to mechanical load (stretch). In the present study, we compared the extent and time course of $p70^{S6k}$ and 4E-BP1 phosphorylation after muscle stretch in isolated fast- and slow-twitch skeletal muscles from different age groups of F344XBN rats. We demonstrate that twenty percent stretch of EDL and soleus muscles in adult animals increases the "ERK 1/2 MAPK"- (Thr 421/Ser 424) dependent phosphorylation of $p70^{S6k}$ and the "mTOR"- (Thr 37/46) dependent phosphorylation of 4E-BP1. Interestingly, this stretch-induced phosphorylation of $p70^{S6k}$ by ERK1/2 MAPK is conserved with aging while the stretch-induced

phosphorylation of 4E-BP1 by mTOR is not (Figures 5A and B and 6A and B). This latter finding suggests that stretch-induced increases in mTOR activity (phosphorylation) are diminished with aging. To examine this possibility we investigated the phosphorylation status of the mTOR Ser2448 residue (Figure 3A and B). This residue is thought to become phosphorylated in response to increases in Akt/PKB activity [13, 14, 16, 27, 152, 153] and was chosen since previous data has suggested that the phosphorylation status of this residue is critical in modulating the activity of mTOR [13, 153]. As shown in Figure 3A, we demonstrate that in adult animals, twenty percent stretch induces the phosphorylation of the mTOR Ser 2448 residue in the fast-twitch EDL but not in the slow-twitch soleus (Figure3B). Our findings of a lack of mTOR Ser 2448 phosphorylation following an increased loading stimulus in the predominantly type I soleus muscle is consistent with a previous study by Parkington and colleagues (2003) who demonstrated similar findings in the soleus following a bout of high intensity concentric contractions. Collectively, these data suggest that the mechanism(s) of load-induced mTOR activation differ between different fiber types or alternatively, that fiber type-dependent activation of mTOR may be unique to the acute nature of the stimulus. Why 4E-BP1 phosphorylation by mTOR is increased in the adult soleus with stretch without a concomitant increase in the activation of mTOR is unknown, but because our data is limited to selected time points they do not allow us to rule out any changes that may have occurred at time points that were not examined in this study. It is possible that if we sampled at other time points that we would have found a stretch-induced changes in the phosphorylation of mTOR. Interestingly, in the very aged EDL we see that increased loading induced decreases in the extent of mTOR phosphorylation (Figure 3A). The physiological ramifications of this pattern of mTOR regulation are not known but suggest that our loading stimulus is not associated with the

activation of protein synthesis in this very aged muscle. Such findings, if present, would be in agreement with previous demonstrating that aging increases the susceptibility of cells to environmental stress [128]. We demonstrate that static stretch decreases the extent of mTOR dependent $p70^{S6k}$ (Thr 389) phosphorylation in the fast-twitch EDL but not in the slow-twitch soleus (Figure 4 A and B). Interestingly, this response is conserved with aging. Why a stretch stimulus would act to decrease $p70^{S6k}$ (Thr 389) phosphorylation in one muscle type but not another is unknown. Taken together, these data suggest that different fiber types respond differently to mechanical stimuli. In addition, our findings that the amount of 4E-BP1 phosphorylated by mTOR is elevated at the same time points of observation where we find no increases in the extent of mTOR phosphorylated $p70^{S6k}$ may indicate that mTOR exhibits different preferences in substrate phosphorylation. Future experimentation will no doubt add to our understanding.

In conclusion, the results of this study have shown significant alterations in the skeletal muscle content and degree of basal phosphorylation of $p70^{S6k}$, mTOR, GSK-3β, 4E-BP1 and SHP-2 with aging. In the adult EDL static stretch increases mTOR phosphorylation however this response is altered with aging. The "ERK1/2 MAPK"- dependent phosphorylation of $p70^{S6k}$ is not affected by aging in either the rat EDL or soleus while conversely, the mTOR dependent activation of 4E-BP1 attenuated. This study underscores the basis for future studies investigated the role that mechanotransduction may play in age-related skeletal muscle signaling.

ACKNOWLEDGEMENTS

This study was supported by National Institute on Aging Grant AG-20370 to Eric Blough and by NSF EPSCOR to Marshall University.

EDL
Table 1

Tissue concentration of mTOR, GSK-3ß, $p70^{S6k}$, 4E-BP1 and SHP-2 in EDL excised from, aged (30 month) and very aged (36 month) in comparison to young (6month) Fischer 344X Brown Norway hybrid rats. Results were obtained by the Immunoblot analysis using antibodies which recognized the unphosphorylated and phosphorylated forms of the proteins. Data are presented as percent of the young adult value. (NC) indicates the no change in the protein levels from the young adult.

EDL	CONTENT	
	30 month	36 month
mTOR	↓(39%)	↓(35%)
GSK-3ß	NC	↓(44%)
$p70^{S6k}$	↓(27%)	↓(58%)
4E-BP1	NC	↓(33%)
SHP-2	↑(38%)	↑(38%)

Soleus

Table 2

Tissue concentration of mTOR, GSK-3ß, $p70^{S6k}$, 4E-BP1 and SHP-2 in soleus excised from, aged (30 month) and very aged (36 month) in comparison to young (6month) Fischer 344X Brown Norway hybrid rats. Results were obtained by the immunoblot analysis using antibodies which recognized the unphosphorylated and phosphorylated forms of the proteins. Data are presented as percent of the young adult value. (NC) indicates the no change in the protein levels from the young adult.

Soleus	CONTENT	
	30 month	36 month
mTOR	NC	↓(48%)
GSK-3ß	↓(24%)	↓(31.5%)
$p70^{S6k}$	↓(38%)	↓(68%)
4E-BP1	↓(58%)	↓(60%)
SHP-2	NC	↓(44.4%)

Table 3: Basal phosphorylation in comparison to young (6month) and phosphorylation with twenty percent stretch at 5 and 15 min in comparison with control with in age group of mTOR, GSK-3ß, $p70^{S6k}$, 4E-BP1 and SHP-2 in EDL excised from young (6month), aged (30 month) and very aged (36 month) and Fischer 344X Brown Norway hybrid rats. Results were obtained by the immunoblot analysis using antibodies which recognized the phosphorylated forms of the proteins. Data are presented as percent of the young adult value. (NC) indicates the no change in the protein levels from the young adult.

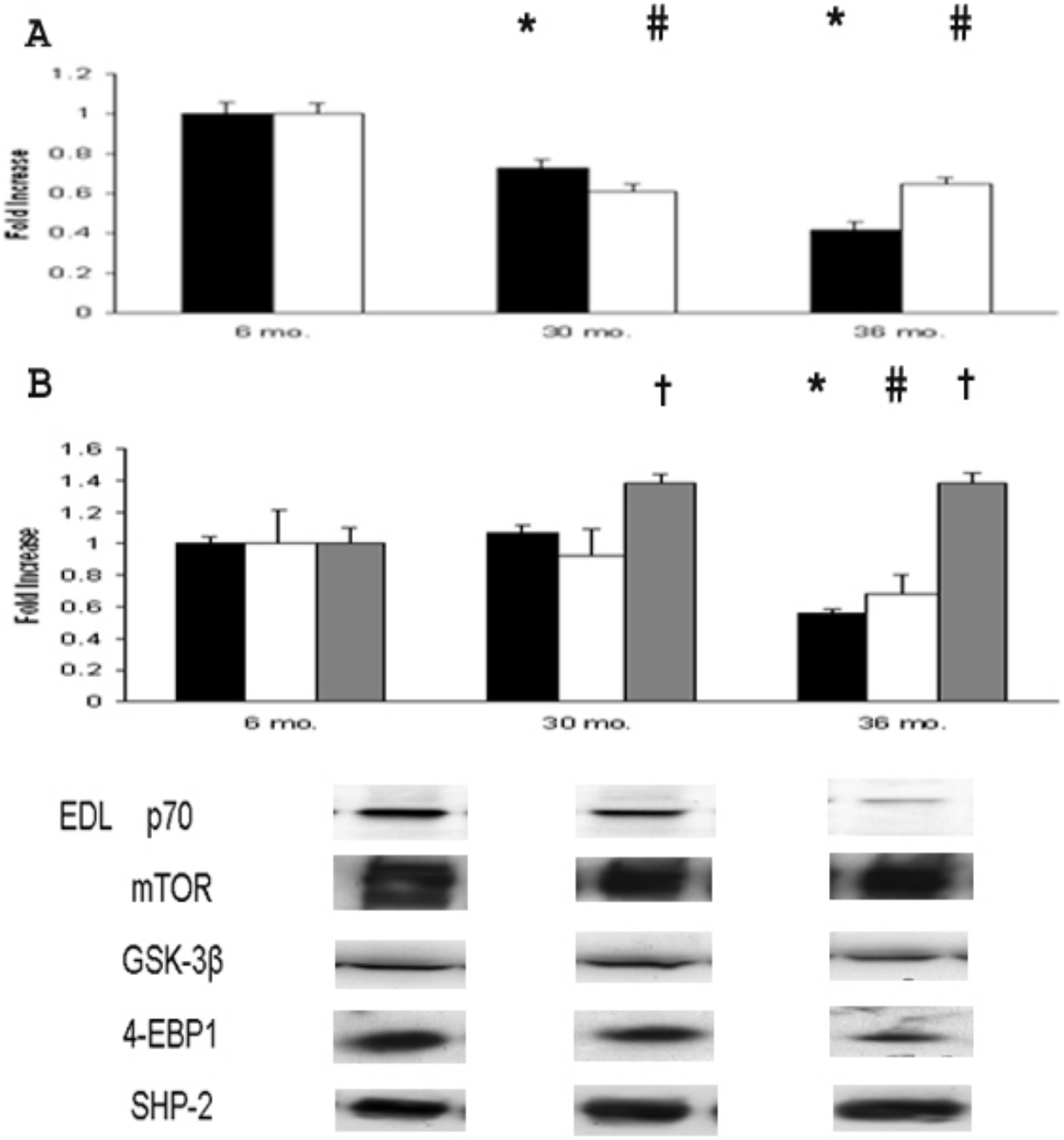
EDL	control	5 min	15min
p-p70(Thr389)			
6month	=	↓(27%)	↓(14.7%)
30 month	↑(14%)	↓(22%)	↓(24.5%)
36 month	NC	↓(21%)	↓(54%)
p-p70(Thr421Ser424)			
6month	=	↑(41.7%)	↑(81.6%)
30 month	NC	NC	↑(52%)
36 month	NC	↑(36.7%)	↑(32%)
p-GSK-3β			
6month	=	NC	↑(7.7%)
30 month	↓(14%)	↓(10.6%)	NC
36 month	↓(36.8%)	↓(12.9%)	↓(9%)
p-SHP-2			
6month	=	NC	NC
30 month	NC	NC	NC
36 month	↓(20.7%)	NC	NC
p-4E-BP1			
6month	=	↑(69%)	↑(99%)
30 month	NC	NC	NC
36 month	↑(80%)	NC	↓(121%)
p-mTOR			
6month	=	NC	↑(100.8%)
30 month	↑(31%)	NC	NC
36 month	↑(219%)	↓(229%)	↓(243%)

Table 4: Basal phosphorylation in comparison to young (6month) and phosphorylation with twenty percent stretch at 5 and 15 min in comparison with control with in age group of mTOR, GSK-3ß, $p70^{S6k}$, 4E-BP1 and SHP-2 in soleus excised from young (6month), aged (30 month) and very aged (36 month) and Fischer 344X Brown Norway hybrid rats. Results were obtained by the immunoblot analysis using antibodies which recognized the phosphorylated forms of the proteins. Data are presented as percent of the young adult value. (NC) indicates the no change in the protein levels from the young adult.

SOLEUS	control	5 min	15min
p-$p70^{S6k}$ (Thr389)			
6month	=	NC	NC
30 month	NC	NC	NC
36 month	NC	NC	NC
p-$p70^{S6k}$ (Thr421Ser424)			
6month	=	NC	NC
30 month	NC	NC	↑(29%)
36 month	↓(23%)	↑(28%)	↑(22%)
p-GSK-3β			
6month	=	↓(25%)	↓(27%)
30 month	↓(29%)	↓(7%)	NC
36 month	↓(31%)	↓(5%)	NC
p-SHP-2			
6month	=	NC	NC
30 month	↑(39%)	↓(22%)	NC
36 month	↓(59%)	↑(10%)	NC
p-4E-BP1			
6month	=	↑(16.8%)	↑(47%)
30 month	↑(19%)	NC	NC
36 month	NC	↓(39.4%)	↓(60.4%)
p-mTOR			
6month	=	NC	↓(21%)
30 month	↑(33%)	↓(40%)	↓(52%)
36 month	↓(44%)	NC	↓(17%)

EDL

Figure 1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1.6
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1.2
1
0.8
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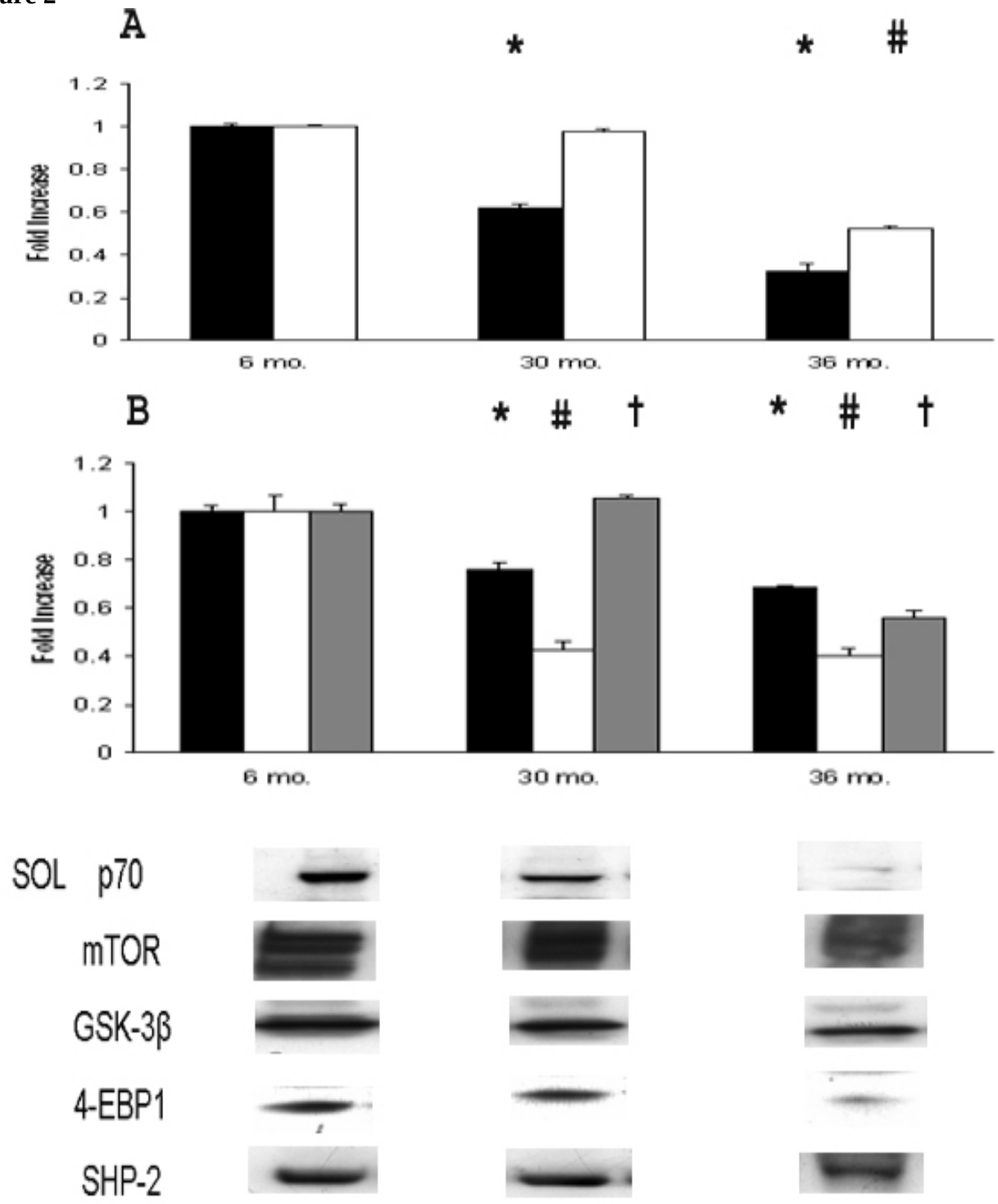
EDL p70
mTOR
GSK-3β
4-EBP1
SHP-2

SOL

Figure 2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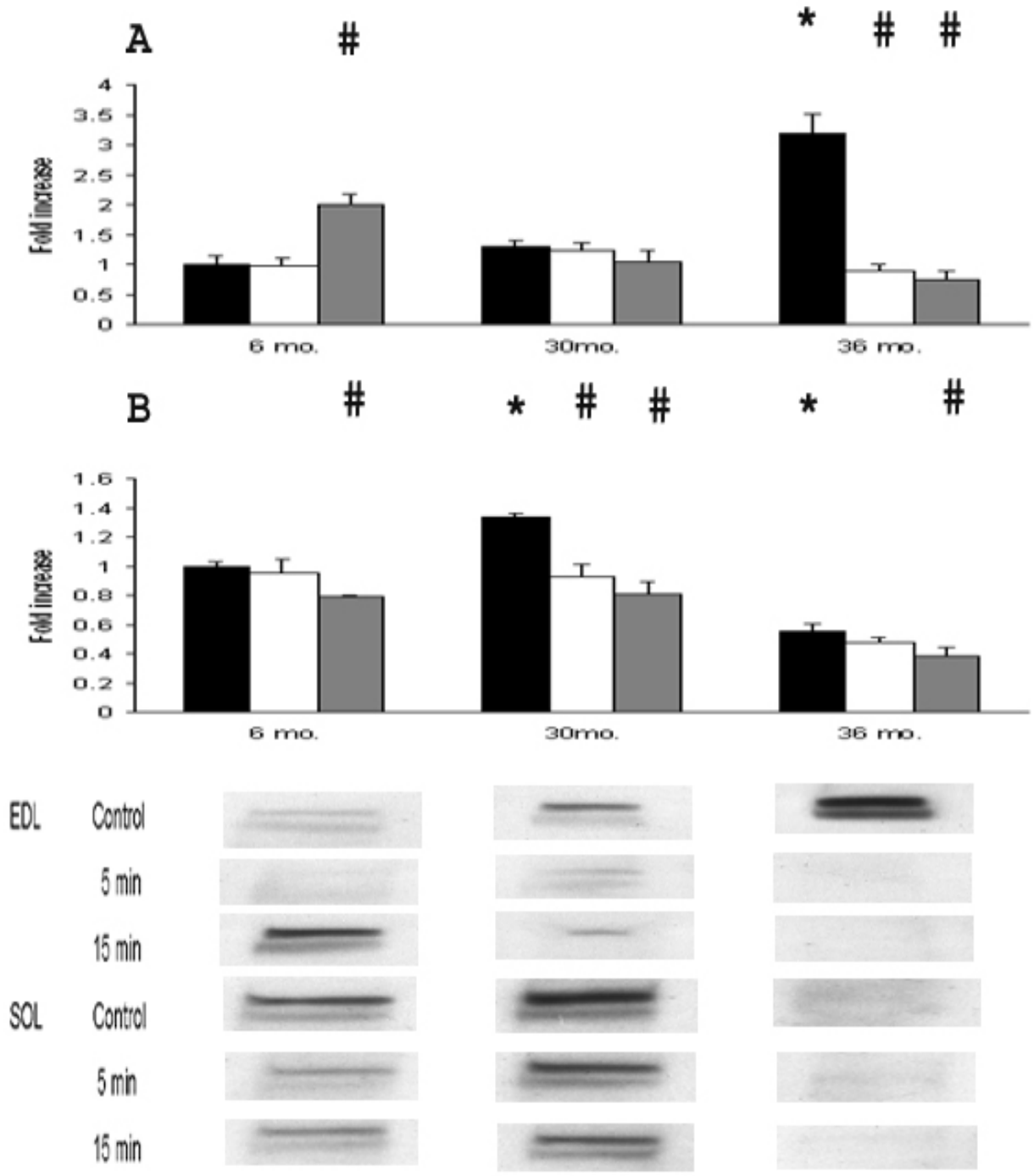
mTOR
GSK-3β
4-EBP1
SHP-2

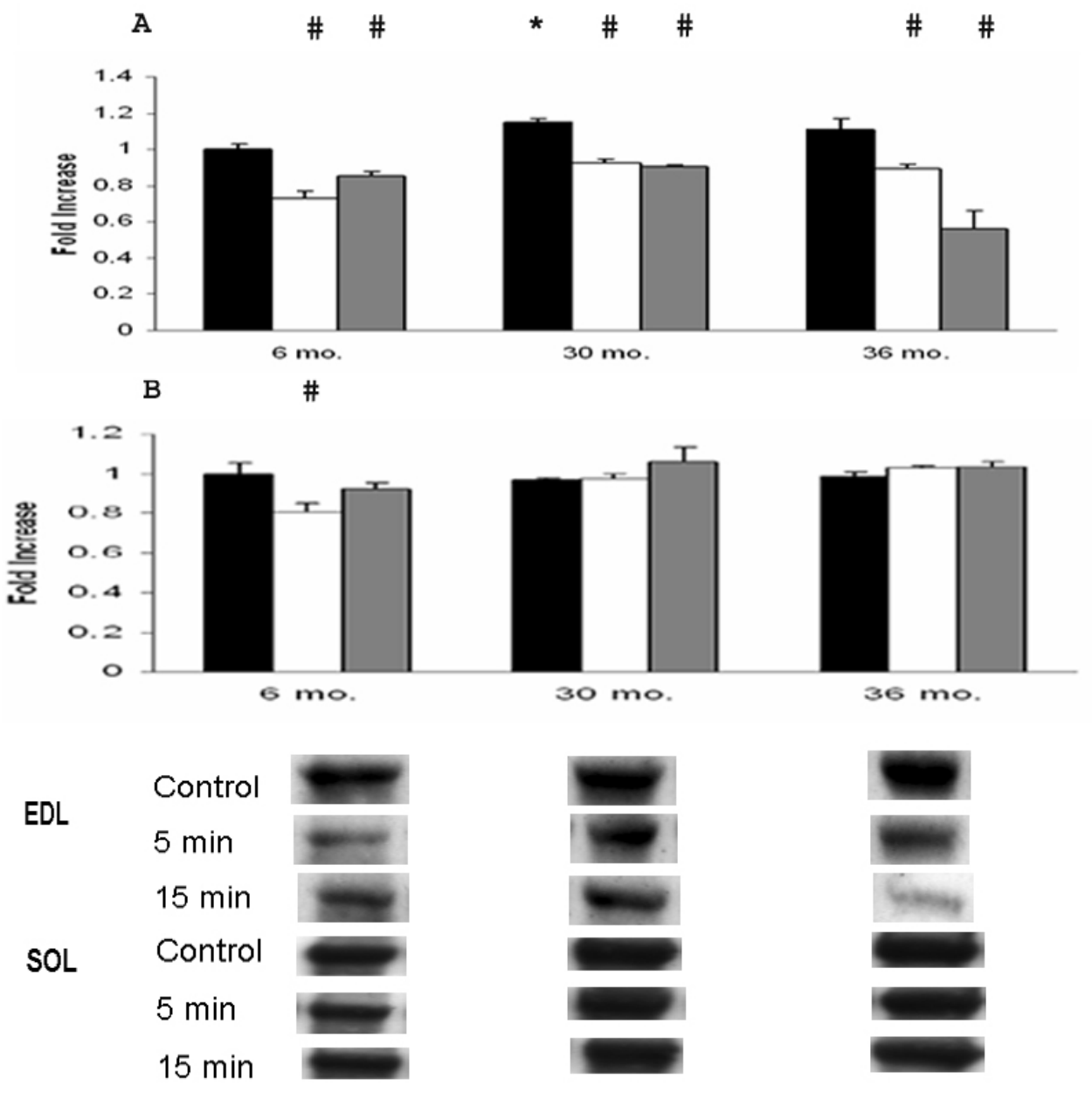
P-mTOR

Figure 3



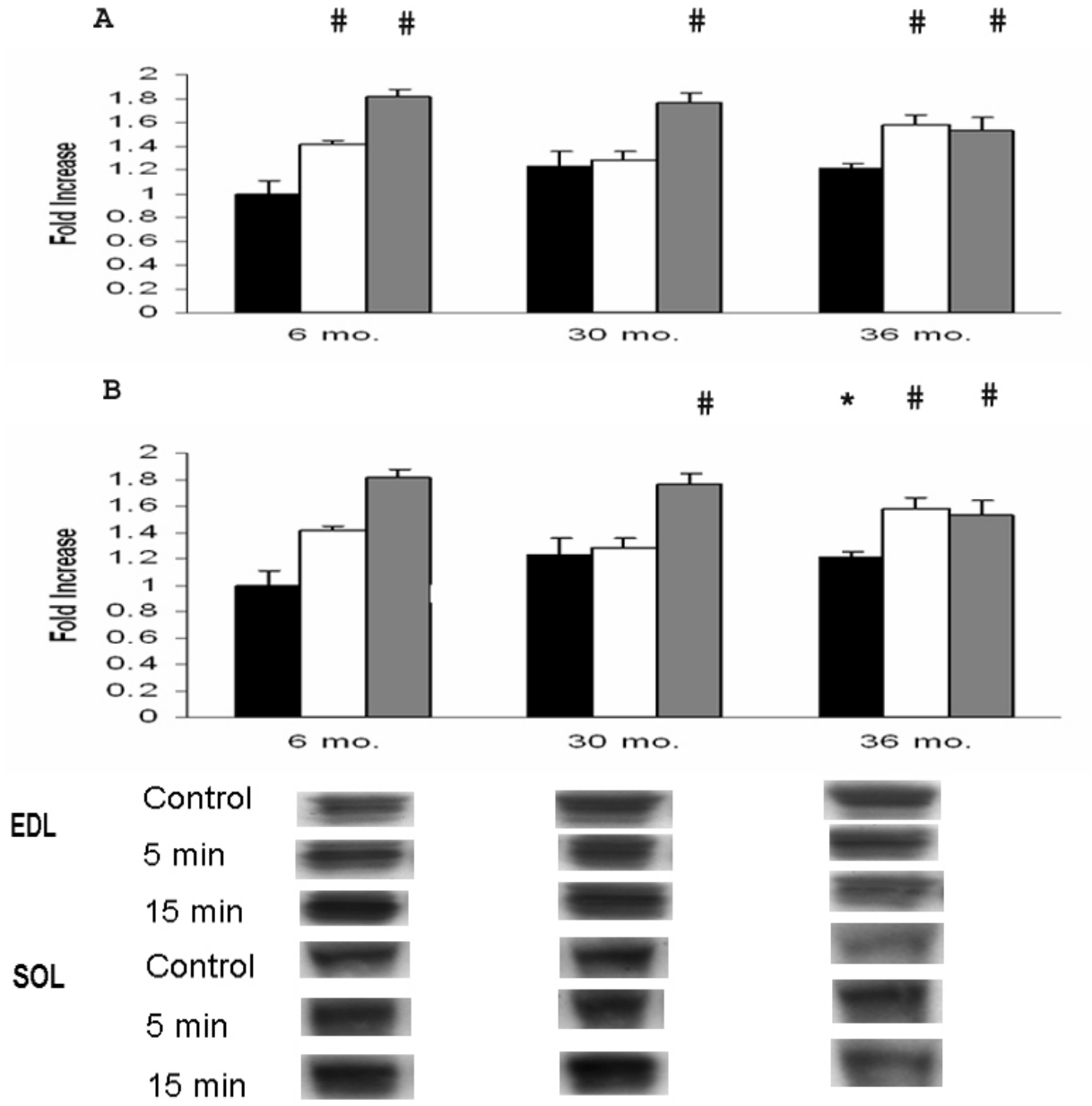
P- $p70^{s6k}$(Thr389)

Figure 4



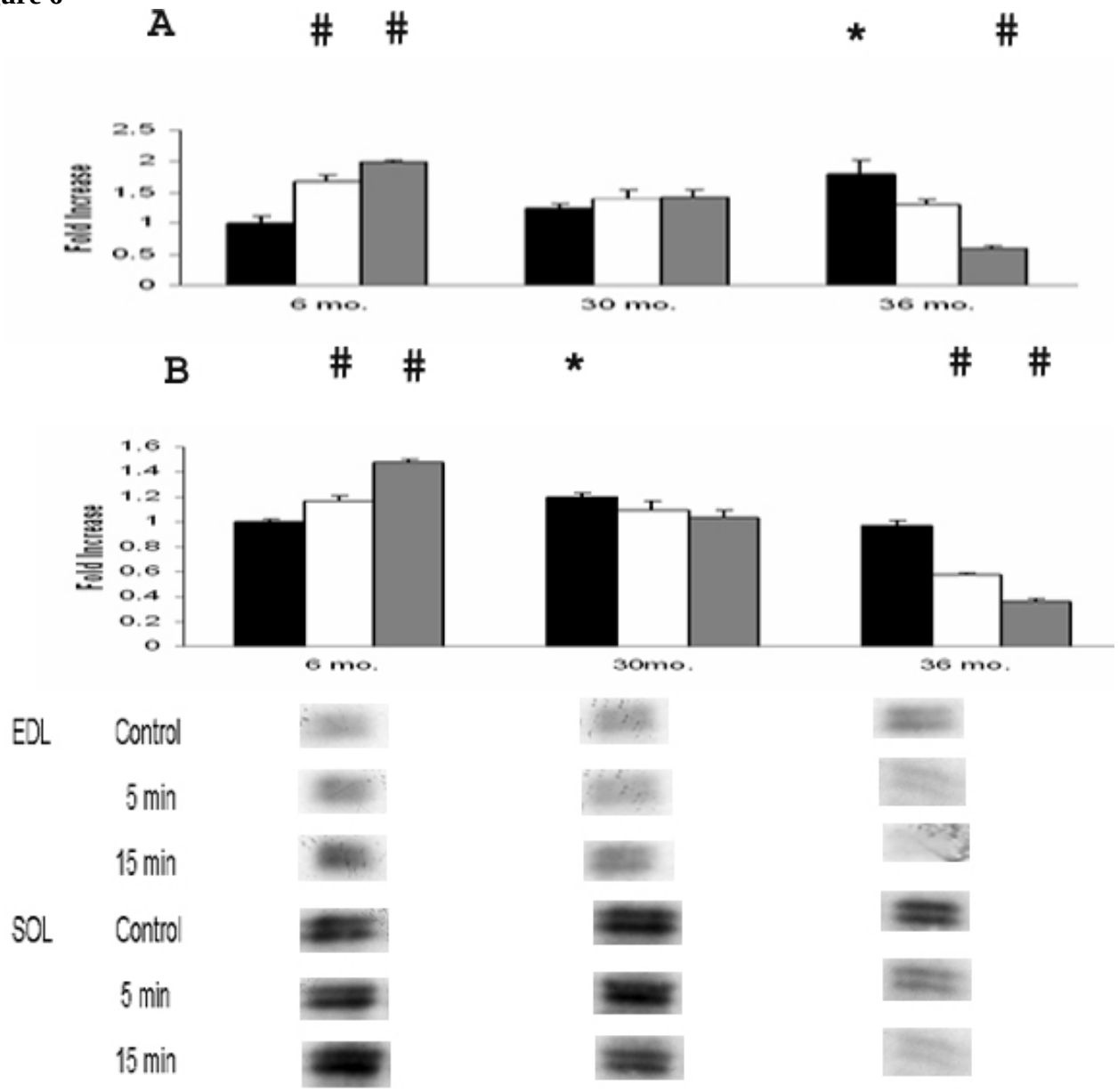
P- $p70^{s6k}$(Thr421/Ser424)

Figure 5



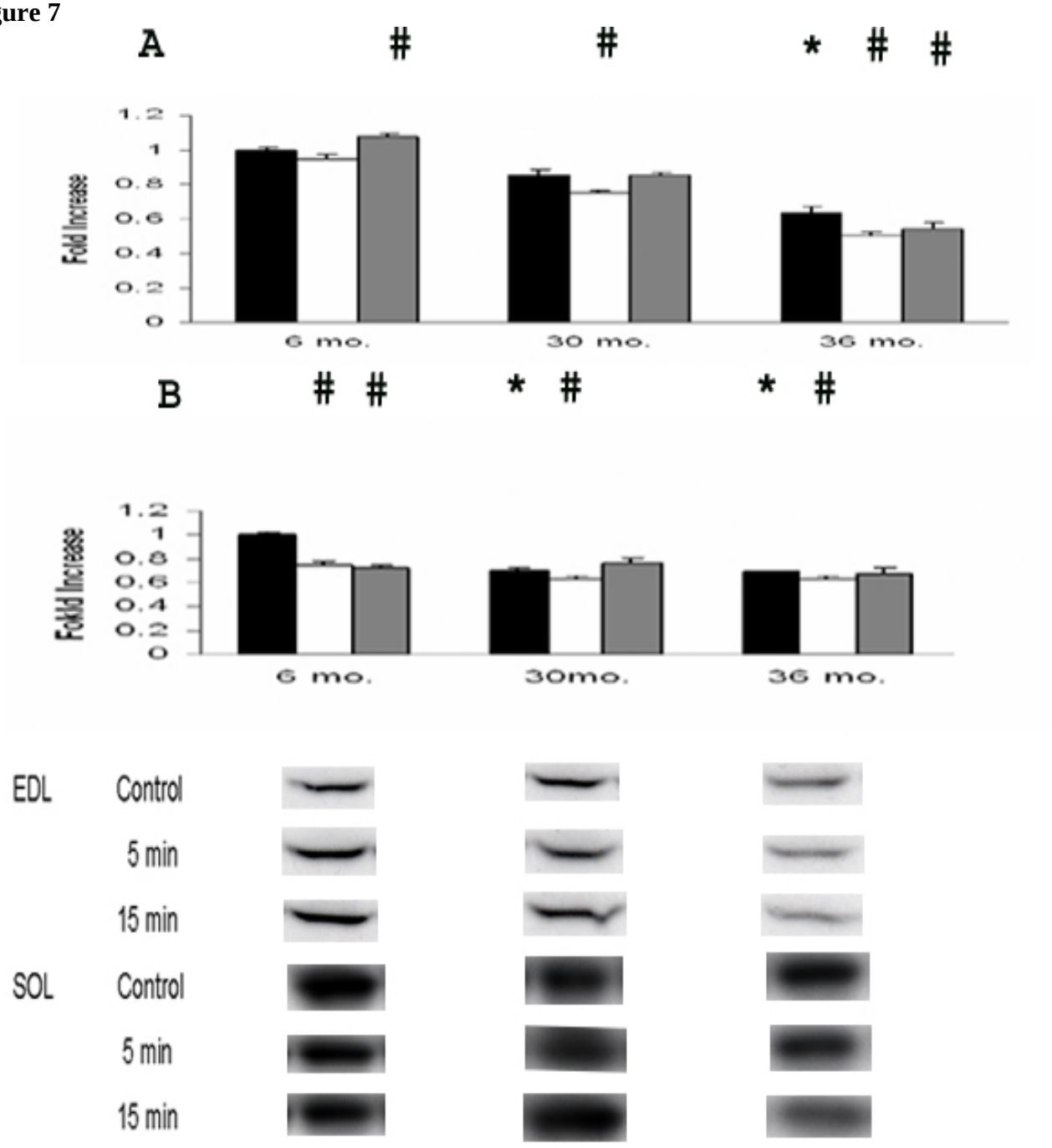
P-4E-BP1

Figure 6



P-GSK-3ß

Figure 7



P-SHP-2

Figure 8

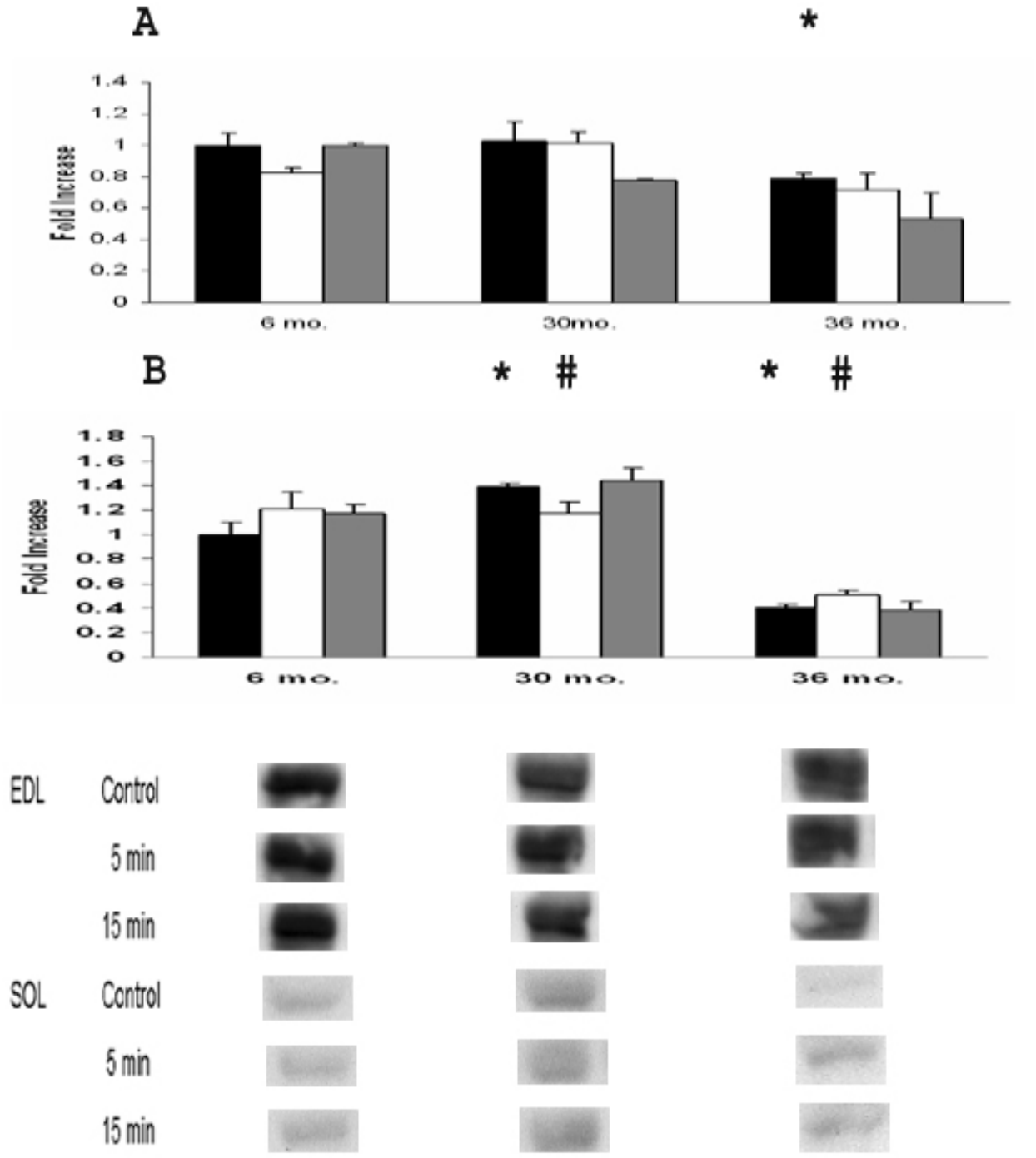


FIGURE LEGENDS

Figure 1. EDL tissue content of: A) $p70^{S6k}$ and mTOR B) GSK-3ß, 4E-BP1 and SHP-2 in EDL from 6 month (young adult), 30 month (aged) and 36 month (very aged) Fischer 344/Brown Norway F1 hybrid rats. Relative changes in protein levels were determined by Western blot analysis. The data are presented as percent of the 6 month value (n = 4). An asterisk, pound or cross indicates significant difference from the corresponding 6 month value, ($p < 0.05$).

Figure 2. Soleus tissue content of: A) $p70^{S6k}$ and mTOR B) GSK-3ß, 4E-BP1 and SHP-2 in soleus muscle from 6 month (young adult), 30 month (aged) and 36 month (very aged) Fischer 344/Brown Norway F1 hybrid rats. Relative changes in protein levels were determined by Western blot analysis. The data are presented as percent of the 6 month value (n = 4). An asterisk, pound or cross indicates significant difference from the corresponding 6 month value, ($p < 0.05$).

Figure 3. The basal (control) and twenty percent stretch-induced phosphorylation at 5 and 15 minutes of the mTOR in A) EDL B) soleus from 6-, 30- and 36- month old rats. Results were obtained by Western blot with immunodetection for mTOR phosphorylated at Thr 2248. Phosphorylation status was calculated as phosphospecific optical density divided by the 6 month value. An asterisk or pound indicates significant difference from the corresponding 6 month value or the control, respectively ($p < 0.05$), n = 4/group.

Figure 4. The basal (control) and twenty percent stretch-induced phosphorylation at 5 and 15 minutes of the "mTOR"- dependent form of $p70^{S6k}$ (Thr 389) in A) EDL B) soleus from 6-, 30- and 36- month old rats. Results were obtained by Western blot with immunodetection for $p70^{S6k}$ phosphorylated at Thr 389. Phosphorylation status was calculated as phosphospecific optical density divided by the 6 month value. An asterisk or pound indicates significant difference from the corresponding 6 month value or the control, respectively ($p < 0.05$), n = 4/group.

Figure 5. The basal (control) and twenty percent stretch-induced phosphorylation at 5 and 15 minutes of the "ERK 1/2 MAPK"- dependent form of $p70^{S6k}$ (Thr 421/Ser 424) in A) EDL B) soleus from 6-, 30- and 36- month old rats. Results were obtained by Western blot with immunodetection for $p70^{S6k}$ phosphorylated at Thr 421/Ser 424. Phosphorylation status was calculated as phosphospecific optical density divided by the 6 month value. An asterisk or pound indicates significant difference from the corresponding 6 month value or the control, respectively ($p < 0.05$), n = 4/group.

Figure 6. Basal (control) and twenty percent stretch-induced phosphorylation at 5 and 15 minutes of "mTOR"- dependent form 4E-BP1 in A) EDL B) soleus from 6, 30, and 36 month old rats. Results were obtained by Western blot with immunodetection of 4E-BP1 phosphorylated at Thr 37/46. Phosphorylation status was calculated as phosphospecific optical density divided by the 6 month value. An asterisk or pound indicates significant difference from the corresponding 6 month value or the control, respectively ($p < 0.05$), n = 4/group.

Figure 7. Basal (control) and twenty percent stretch-induced phosphorylation at 5 and 15 minutes of GSK-3ß in A) EDL B) soleus from 6-, 30-, and 36- month old rats. Results were obtained by Western blot with immunodetection of GSK-3ß phosphorylated at Ser 9. Phosphorylation status was calculated as the phosphospecific optical density divided by the 6 month value. An asterisk or pound indicates significant difference from the corresponding 6 month value or the control, respectively ($p < 0.05$), n = 4/group.

Figure 8. Basal (control) and twenty percent stretch-induced phosphorylation at 5 and 15 minutes of SHP-2 in A) EDL B) soleus from 6-, 30-, and 36- month old rats. Results were obtained by Western blot with immunodetection of SHP-2 phosphorylated on Tyr 542. Phosphorylation status was calculated as phosphospecific optical density divided by the 6 month value. An asterisk or pound indicates significant difference from the corresponding 6 month value or the control, respectively ($p < 0.05$), n = 4/group.

Chapter 4

CONCLUSIONS

1. Aging was found to significantly decrease the soleus and EDL content of mTOR, $p70^{S6k}$, GSK-3ß and 4E-BP1. Conversely, aging increased the content of SHP-2 in the EDL, but decreased SHP-2 content in the soleus.
2. Aging significantly increased the basal phosphorylation of p- $p70^{S6k}$ (T389) and 4E-BP1 in the EDL . In the soleus, aging decreased the basal phosphorylation of p- $p70^{S6k}$ (421/424). The basal phosphorylation of GSK-3ß and SHP-2 decreased with age in both the EDL and soleus.
3. Twenty percent stretch of the EDL muscle increased the phosphorylation of 4E-BP1 at 5 and 15 minutes and mTOR and GSK-3ß at 15 minutes in the younger but not in the aged or very aged EDL muscles. However, SHP-2 phosphorylation with stretch did not change in all the three age groups. On the contrary, mTOR mediated phosphorylation of $p70^{S6k}$ (THR 389) decreased in all the three age groups and ERK1/2 mediated phosphorylation of $p70^{S6k}$ (THR421/SER424) increased in all the three age groups with twenty percent stretch. Phosphorylation of 4E-BP1 at 5 and 15 min with twenty percent stretch of soleus muscle decreased with age however, mTOR mediated phosphorylation of $p70^{S6k}$ (THR 389) did not change. Conversely, ERK1/2 mediated phosphorylation of $p70^{S6k}$ (THR421/SER424) and phosphorylation of SHP-2 with stretch stimulus increased with age. Phosphorylation of mTOR and GSK-3ß decreases with stretch in all the three age groups.

FURTHER DIRECTIONS

Future directions for research based on this study should focus on the mechanisms associated with the differences in twenty percent stretch induced $p70^{s6k}$ and 4E-BP1 activation with aging in the F344BN EDL and soleus muscles.

$p70^{s6k}$ and 4E-BP1 activation is thought to require the assimilation of many different upstream participants like PDK, AKT/PKB, mTOR, PKC zeta and MAPK's [14, 16, 25, 27, 28, 106, 153-159]. An altered activation of $p70^{s6k}$ and 4E-BP1 with twenty percent stretch in the aged may be due to differences in the amount of $p70^{s6k}$ and 4E-BP1 associated of PDK, AKT/PKB mTOR PKC zeta or MAPK's. Immunoprecipitation experiments using antibodies to any of these molecules and then probing immunoprecipatated complexes for differences in the amount of $p70^{s6k}$ and 4E-BP1 with aging would address this speculation.

Additionally, the present study demonstrates an increased basal phosphorylation of mTOR, $p70^{s6k}$ and 4E-BP1 with aging in the soleus and EDL muscles. We speculate that these findings could be due to an increased oxidative stress in the older muscles. Indeed, previous studies by Marzani *et al.* (2004) have demonstrated increases in the amount of reactive oxygen species (ROS) with aging in muscle [128]. Whether or not increased ROS might be responsible for age-associated increases in the basal phosphorylation of mTOR, $p70^{s6k}$ and 4E-BP1 with aging could be determined by measuring the amount of ROS present with aging and then employing ROS scavengers to examine whether this might decrease the phosphorylation of these proteins.

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APPENDIX

$p70^{s6k}$ EDL

Film properties report $p70^{s6k}$ EDL

Experimenter: Sreevani Uddemarri Muscle : EDLSpecies: F344 X BN hybrid rat

Protein concentration: 60 µg/ml Gel type: 7.5% Tris-HCL SDSPAGE

Electrophoresis Voltage: 125V Transfer Voltage: 24V Duration: 45 min

Primary Antibody: $p70^{s6k}$(Cell Signaling) Primary Antibody Dilution: 1:1000

Incubation Time: overnight @ 4°C Medium: 5% BSA Secondary Antibody: Anti Rabbit

Secondary Antibody Dilution: 1:1000 Incubation Time: 1hr @ room temp

Medium: 5% milk in TBS-T Exposure Time 3 min Molecular weight: 70 kDa

Lane 1: Rainbow Marker RPN756 3µl

Lane 2: 6 month control

Lane 3: 6 month 5 min

Lane 4: 6 month 15 min

Lane 5: 30 month control

Lane 6: 30 month 5 min

Lane 7: 30 month 15min

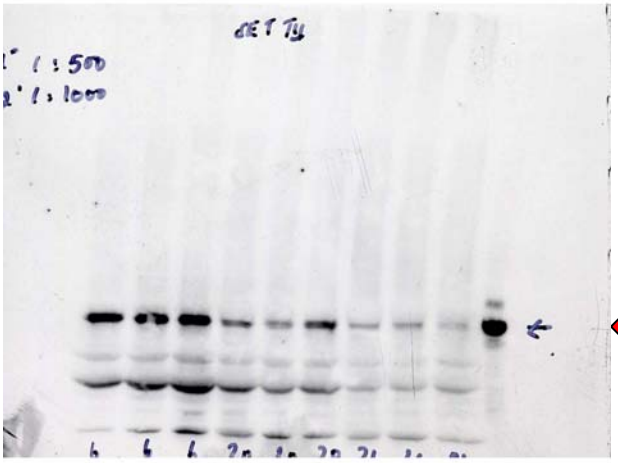
Lane 8: 36 month control

Lane 9: 36 month 5 min

Lane 10: 36 month 15 min

Lane 11: NIH 3T3 Activated Cell Extract 3µl

Lane 12: Biotinylated Ladder 3 µl



Raw data

This section represents the raw data tables produced from spot densitometry of the immunoblot films.

$p70^{s6k}$ in EDL Data set

Raw IOD values (relative percentage)

	6 month	**30 month**	**36 month**
Raw values	36.2	36.7	27.1
	42.6	43	14.5
	43.2	42.8	14
	42.2	32.8	25
	42.6	31.1	26.2
	40	35.4	24.6
	58.2	27.4	14.4
	57.8	27.8	14.4
	57.3	28.1	14.6
N	9	9	9
Mean	**46.67778**	**33.9**	**19.42222**
Strandard Deviation	8.581051	6.064858	6.022204
Standard Error of the mean	**3.03386**	**2.144251**	**2.129171**
% Relative expression	**100**	**72.62557**	**41.60914**
SE	6.499581	4.59373	4.561423

Statistics
One Way Analysis of Variance
Normality Test: Failed (P = 0.003)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0.05
6 months vs 36 months	151.000	6.341	Yes
6 months vs 30 months	59.000	2.478	No
30 months vs 36 months	92.000	3.864	Yes

p-p70^{s6k} EDL

Film properties report p-p70^{s6k}(Thr389) EDL

Experimenter: Sreevani Uddemarri Muscle : EDL Species: F344 X BN hybrid rat

Protein concentration: 60 µg/ml Gel type: 7.5% Tris-HCL SDSPAGE

Electrophoresis Voltage: 125V Transfer Voltage: 24V Duration: 45 min

Primary Antibody: p- p70^{s6k}(Thr389)(Cell Signaling)Primary Antibody Dilution: 1:1000

Incubation Time: overnight @ 4°C Medium: 5% BSA

Secondary Antibody: Anti Rabbit Secondary Antibody Dilution: 1:1000

Incubation Time: 1hr @ room temp Medium: 5% milk in TBS-T

Exposure Time 3 min Molecular weight: 70 kDa

Lane 1: Rainbow Marker RPN756 3µl

Lane 2: 6 month control

Lane 3: 6 month 5 min

Lane 4: 6 month 15 min

Lane 5: 30 month control

Lane 6: 30 month 5 min

Lane 7: 30 month 15min

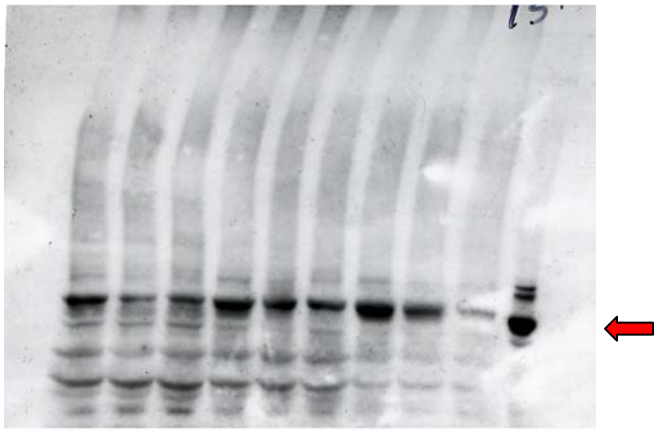
Lane 8: 36 month control

Lane 9: 36 month 5 min

Lane 10: 36 month 15 min

Lane 11: NIH 3T3 Activated Cell Extract 3µl

Lane 12: Biotinylated Ladder 3 µl



Raw data

This section represents the raw data tables Produced from spot densitometry of the immunoblot films.

p-p70^{s6k}(Thr389) in EDL Data set

Raw IOD values (relative percentage)

	6 Control	**6 5 min**	**6 15 min**	**30 Control**	**30 5 min**	**30 15 min**	**36 Control**	**36 5 min**	**36 15 min**
Raw values	12.2	10.4	11	14.2	11.2	10.9	11	10.1	9.1
	13	8.6	10.9	14.5	11.8	10.9	14.8	11.6	4.1
	13.1	8.3	9.8	14.4	11.8	11.1	15.4	11.8	4.4
	12.7	7.5	11.3	14.9	11.6	11.3	15	11.5	4.2
	11.6	10.3	10.4	13	10.3	11.1	12.6	11	9.7
	11.3	8.8	9.6	13.9	11.8	11.5	13.1	10.2	9.8
N	6	6	6	6	6	6	6	6	6
Mean	**12.31667**	**8.983333**	**10.5**	**14.15**	**11.41667**	**11.13333**	**13.65**	**11.03333**	**6.883333**
Strandard Deviation	0.746771	1.147897	0.687023	0.653452	0.594699	0.233809	1.710848	0.733939	2.91439
Standard Error of the mean	**0.333966**	**0.513355**	**0.307246**	**0.292233**	**0.265957**	**0.104563**	**0.765114**	**0.328228**	**1.303355**
Relative Expression Level	**100**	**0.729364**	**0.852503**	**1.14885**	**0.926928**	**0.903924**	**1.108254**	**0.895805**	**0.558863**
Standard error of the mean	0.027115	0.04168	0.024946	0.023727	0.021593	0.00849	0.06212	0.026649	0.10582

Statistics
One Way Analysis of Variance
Normality Test: Passed (P = 0.016)
Equal Variance Test: Failed (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0.05
30 Control vs 36 15 min	287.500	6.721	Yes
30 Control vs 6 5 min	266.000	6.218	Yes
30 Control vs 6 15 min	197.500	4.617	Yes
30 Control vs 6 15 min	197.500	4.617	Yes
30 Control vs 36 5 min	146.000	3.413	No
30 Control vs 30 15 min	145.500	3.401	Do Not Test
30 Control vs 30 5 min	105.500	2.466	Do Not Test
30 Control vs 6 Control	56.000	1.309	Do Not Test
30 Control vs 36 Control	23.500	0.549	Do Not Test
36 Control vs 36 15 min	264.000	6.171	Yes
36 Control vs 6 5 min	242.500	5.669	Yes
36 Control vs 6 15 min	174.000	4.067	No
36 Control vs 6 15 min	174.000	4.067	Do Not Test
36 Control vs 36 5 min	122.500	2.864	Do Not Test
36 Control vs 30 15 min	122.000	2.852	Do Not Test
36 Control vs 30 5 min	82.000	1.917	Do Not Test
36 Control vs 6 Control	32.500	0.760	Do Not Test
6 Control vs 36 15 min	231.500	5.412	Yes
6 Control vs 6 5 min	210.000	4.909	Yes

Film properties report p- p70^{s6k}(Thr421Ser424) EDL

Experimenter: Sreevani Uddemarri

Muscle : EDL Species: F344 X BN hybrid rat

Protein concentration: 60 µg/ml Gel type: 7.5% Tris-HCL SDSPAGE

Electrophoresis Voltage: 125V Transfer Voltage: 24V Duration: 45 min

Primary Antibody: p- p70^{s6k}(Thr421Ser424)(Cell Signaling) Primary Antibody Dilution: 1:1000

Incubation Time: overnight @ 4°C Medium: 5% BSA

Secondary Antibody: Anti Rabbit Secondary Antibody Dilution: 1:1000

Incubation Time: 1hr @ room temp Medium: 5% milk in TBS-T

Exposure Time 3 min Molecular weight: 70 kDa

Lane 1: Rainbow Marker RPN756 3µl

Lane 2: 6 month control

Lane 3: 6 month 5 min

Lane 4: 6 month 15 min

Lane 5: 30 month control

Lane 6: 30 month 5 min

Lane 7: 30 month 15min

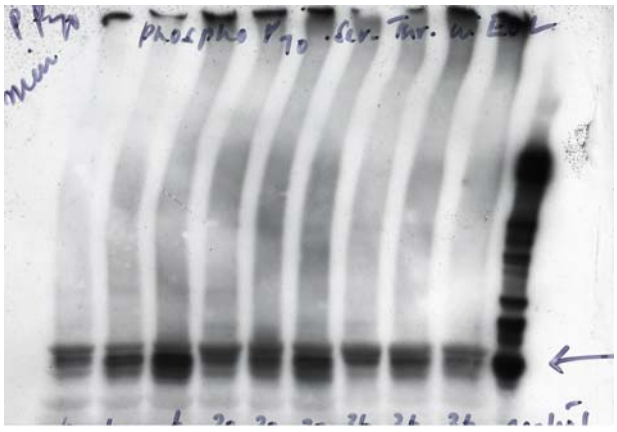
Lane 8: 36 month control

Lane 9: 36 month 5 min

Lane 10: 36 month 15 min

Lane 11: NIH 3T3 Activated Cell Extract 3µl

Lane 12: Biotinylated Ladder 3 µl



Raw data

This section represents the raw data tables produced from spot densitometry of the immunoblot films.

p- $p70^{s6k}$(Thr421Ser424) in EDL Data set

Raw IOD values (relative percentage)

	6 Control	**6 5 min**	**6 15 min**	**30 Control**	**30 5 min**	**30 15 min**	**36 Control**	**36 5 min**	**36 15 min**
Raw values	9.6	11.2	12.9	11.2	11.2	12.4	10	10.9	10.7
	9.3	10.8	13.4	11.3	11.2	12.3	9.8	11.3	10.8
	9.8	11	13.4	12	11.2	12.4	9.7	10.9	9.6
	6.3	11.7	14.9	8	9.4	14.5	8.1	13.5	13.5
	5.5	10.4	15.5	7.1	8.4	15.3	9.6	14.1	14.1
	6.3	11.2	14.9	8.1	8.4	15.5	9.4	13.1	13
N	6	6	6	6	6	6	6	6	6
Mean	7.8	11.05	14.16667	9.616667	9.966667	13.73333	9.433333	12.3	11.95
Strandard Deviation	1.96367	0.437035	1.061446	2.110371	1.399524	1.534492	0.68313	1.431084	1.818516
Standard Error of the mean	0.87818	0.195448	0.474693	0.943787	0.625886	0.686246	0.305505	0.64	0.813265
Relative Expression Level	100	141.6667	181.6239	123.2906	127.7778	176.0684	120.9402	157.6923	153.2051
Standard error of the mean	11.2587	2.5057	6.0858	1.20998	8.0242	8.798	3.9167	8.2051	10.4265

Statistics

One Way Analysis of Variance

Normality Test: Passed (P = 0.011)
Equal Variance Test: Passed (P = 0.099)

Group Name	N	Missing	Mean	Std Dev	SEM
6 Control	6	0	19.104	5.335	2.178
6 Control	6	0	19.104	5.335	2.178
6 5 min	6	0	26.982	2.400	0.980
6 15 min	6	0	34.509	2.625	1.072
30 Control	6	0	25.823	3.060	1.249
30 5 min	6	0	27.022	2.342	0.956
30 15 min	6	0	38.092	8.773	3.582
36 Control	6	0	46.837	16.852	6.880
36 5 min	6	0	59.513	15.165	6.191
36 15 min	6	0	57.200	12.510	5.107

Source of Variation	DF	SS	MS	F	P
Between Groups	9	11756.559	1306.284	15.703	<0.001
Residual	50	4159.357	83.187		
Total	59	15915.915			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

$p70^{s6k}$ Soleus

Film properties report $p70^{s6k}$ Soleus

Experimenter: Sreevani Uddemarri

Muscle : Soleus Species: F344 X BN hybrid rat

Protein concentration: 60 µg/ml Gel type: 7.5% Tris-HCL SDSPAGE

Electrophoresis Voltage: 125V Transfer Voltage: 24V Duration: 45 min

Primary Antibody: $p70^{s6k}$(Cell Signaling) Primary Antibody Dilution: 1:1000

Incubation Time: overnight @ 4°C Medium: 5% BSA

Secondary Antibody: Anti Rabbit Secondary Antibody Dilution: 1:1000

Incubation Time: 1hr @ room temp Medium: 5% milk in TBS-T

Exposure Time 20 min Molecular weight: 70 kDa

Lane 1: Rainbow Marker RPN756 3µl

Lane 2: 6 month control

Lane 3: 6 month 5 min

Lane 4: 6 month 15 min

Lane 5: 30 month control

Lane 6: 30 month 5 min

Lane 7: 30 month 15min

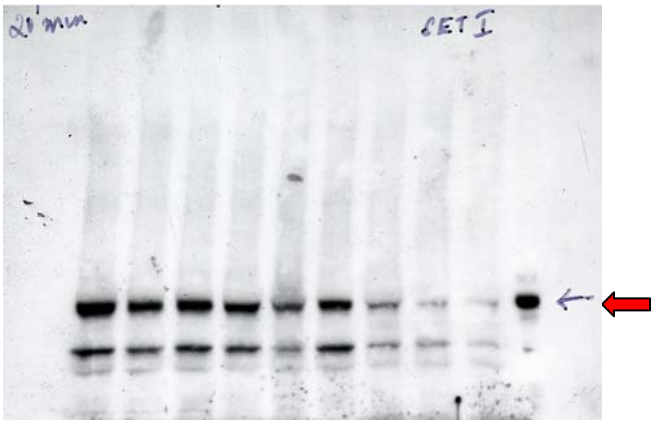
Lane 8: 36 month control

Lane 9: 36 month 5 min

Lane 10: 36 month 15 min

Lane 11: NIH 3T3 Activated Cell Extract 3µl

Lane 12: Biotinylated Ladder 3 µl



Raw data

This section represents the raw data tables produced from spot densitometry of the immunoblot films.

$p70^{s6k}$ in Soleus Data set

Raw IOD values (relative percentage)

	6 month	30 month	36 month
Raw values	48.3	34.1	17.6
	48	34.4	17.6
	47.9	34	18.1
	45	38.4	16.6
	44.7	38.6	16.7
	42.9	39.4	17.7
	61.9	23.8	14.3
	60.7	23.8	15.6
	64.4	21.5	14.1
N	**9**	**9**	**9**
Mean	**51.53333**	**32**	**16.47778**
Strandard Deviation	8.344909	7.054963	1.494806
Standard Error of the mean	**3.03386**	**2.144251**	**2.129171**
% Relative expression	**100**	**62.09573**	**31.97499**
SE	**5.72517**	**4.84018**	**1.025538**

Statistics
One Way Analysis of Variance

Normality Test: Passed (P > 0.200)
Equal Variance Test: Passed (P = 0.057)

Group Name	N	Missing	Mean	Std Dev	SEM
6 months	9	0	51.533	8.345	2.782
30 months	9	0	32.000	7.055	2.352
36 months	9	0	16.478	1.495	0.498

Source of Variation	DF	SS	MS	F	P
Between Groups	2	5554.147	2777.074	68.488	<0.001
Residual	24	973.156	40.548		
Total	26	6527.303			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures Tukey Test:

Comparison	Diff of Means	p	q	P	P<0.050
6 months vs. 36 months	35.056	3	16.516	<0.001	Yes
6 months vs. 30 months	19.533	3	9.203	<0.001	Yes
30 months vs. 36 months	15.522	3	7.313	<0.001	Yes

p- p70^{s6k} Soleus

Film properties report p- p70^{s6k}(Thr389) Soleus

Experimenter: Sreevani Uddemarri

Muscle : Soleus Species: F344 X BN hybrid rat

Protein concentration: 60 µg/ml Gel type: 7.5% Tris-HCL SDSPAGE

Electrophoresis Voltage: 125V Transfer Voltage: 24V Duration: 45 min

Primary Antibody: p- p70^{s6k}(Thr389)(Cell Signaling) Primary Antibody Dilution: 1:1000

Incubation Time: overnight @ 4°C Medium: 5% BSA

Secondary Antibody: Anti Rabbit Secondary Antibody Dilution: 1:1000

Incubation Time: 1hr @ room temp Medium: 5% milk in TBS-T

Exposure Time 3 min Molecular weight: 70 kDa

Lane 1: Rainbow Marker RPN756 3µl

Lane 2: 6 month control

Lane 3: 6 month 5 min

Lane 4: 6 month 15 min

Lane 5: 30 month control

Lane 6: 30 month 5 min

Lane 7: 30 month 15min

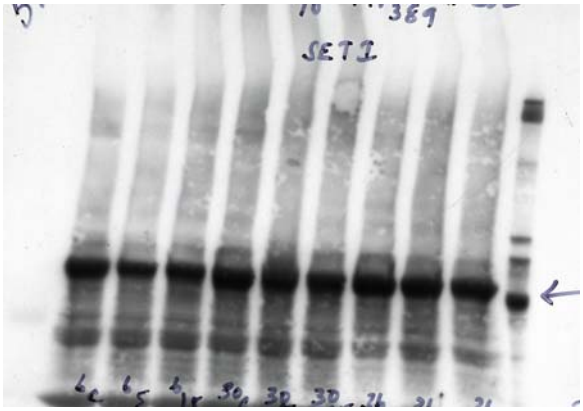
Lane 8: 36 month control

Lane 9: 36 month 5 min

Lane 10: 36 month 15 min

Lane 11: NIH 3T3 Activated Cell Extract 3µl

Lane 12: Biotinylated Ladder 3 µl



Raw data

This section represents the raw data tables Produced from spot densitometry of the immunoblot films.

p- $p70^{s6k}$(Thr389) in soleus Data set

Raw IOD values (relative percentage)

	6 Control	**6 5 min**	**6 15 min**	**30 Control**	**30 5 min**	**30 15 min**	**36 Control**	**36 5 min**	**36 15 min**
Raw values	12.5	9.8	11.3	11.1	10.9	10.6	10.9	11.5	11.3
	13	10.5	10.7	10.3	10.4	10.2	10.6	11.3	12.9
	12.4	10.4	11.4	11.3	10.7	10	10.7	11.5	11.6
	9.9	7.8	9.9	11	11.7	14.5	11.7	11.6	11.9
	10.2	8.2	10.3	11.2	11.6	14.1	11.5	11.9	11
	10.4	8.4	9.7	11.1	11.3	12.8	12	12.3	12.1
N	6	6	6	6	6	6	6	6	6
Mean	11.4	9.183333	10.55	11	11.1	12.03333	11.23333	11.68333	11.8
Strandard Deviation	1.3755	1.190658	0.709225	0.357771	0.517687	2.024516	0.578504	0.360093	0.669328
Standard Error of the mean	0.615142	0.532478	0.317175	0.16	0.231517	0.905391	0.258715	0.161038	0.299333
Relative Expression Level	100	80.5556	92.5439	96.4912	97.3684	105.5556	98.538	102.4854	103.5088
Standard error of the mean	5.396	4.6709	2.7822	1.4035	2.0308	7.942	2.2694	1.4126	2.6257

Statistics
One Way Analysis of Variance
Normality Test: Passed (P > 0.200)
Equal Variance Test: Failed (P = <0.001)
Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
6 control	6	0	11.400	10.200	12.500
6 5 min	6	0	9.100	8.200	10.400
6 15 min	6	0	10.500	9.900	11.300
30 control	6	0	11.100	11.000	11.200
30 5 min	6	0	11.100	10.700	11.600
30 15 min	6	0	11.700	10.200	14.100
36 control	6	0	11.200	10.700	11.700
36 5 min	6	0	11.550	11.500	11.900
36 15 min	6	0	11.750	11.300	12.100

H = 20.052 with 8 degrees of freedom. (P = 0.010)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.010)

Film properties report p- p70^{s6k}(Thr421Ser424) Soleus

Experimenter: Sreevani Uddemarri

Muscle : Soleus Species: F344 X BN hybrid rat

Protein concentration: 60 µg/ml Gel type: 7.5% Tris-HCL SDSPAGE

Electrophoresis Voltage: 125V Transfer Voltage: 24V Duration: 45 min

Primary Antibody: p- p70^{s6k}(Thr421Ser424)(Cell Signaling)Primary Antibody Dilution: 1:1000

Incubation Time: overnight @ 4°C Medium: 5% BSA

Secondary Antibody: Anti Rabbit Secondary Antibody Dilution: 1:1000

Incubation Time: 1hr @ room temp Medium: 5% milk in TBS-T

Exposure Time 3 min Molecular weight: 70 kDa

Lane 1: Rainbow Marker RPN756 3µl

Lane 2: 6 month control

Lane 3: 6 month 5 min

Lane 4: 6 month 15 min

Lane 5: 30 month control

Lane 6: 30 month 5 min

Lane 7: 30 month 15min

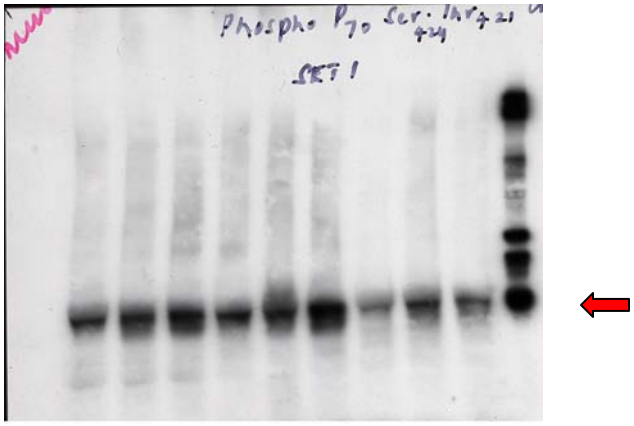
Lane 8: 36 month control

Lane 9: 36 month 5 min

Lane 10: 36 month 15 min

Lane 11: NIH 3T3 Activated Cell Extract 3µl

Lane 12: Biotinylated Ladder 3 µl



Raw data

This section represents the raw data tables Produced from spot densitometry of the immunoblot films.

p-p70^{s6k}(Thr421Ser424) in Soleus Data set

Raw IOD values (relative percentage)

	6 Control	**6 5 min**	**6 15 min**	**30 Control**	**30 5 min**	**30 15 min**	**36 Control**	**36 5 min**	**36 15 min**
Raw values	10.9	12	13.2	10	11.6	12.7	8	11.7	10
	10.4	11.4	10.5	11.6	11.6	14.9	8.9	10.1	10.6
	10.8	11.6	13.1	10.3	11.5	14.5	7.1	11.5	9.7
	11.6	11.1	10.1	11.3	10.1	13.3	9.1	11.8	11.6
	10.8	10.6	11	10.3	10.4	15.3	8.9	10.8	12
	10.4	11.7	12.5	10.6	11.9	12.2	7.9	12.4	10.4
N									
Mean	6	6	6	6	6	6	6	6	6
Strandard Deviation	10.81667	11.4	11.73333	10.68333	11.18333	13.81667	8.316667	11.38333	10.71667
Standard Error of the mean	0.440076	0.493964	1.36626	0.630608	0.741395	1.262405	0.780812	0.813429	0.904249
Relative Expression Level	100	105.3929	108.4746	98.7673	103.3898	127.735	76.8875	105.2388	99.0755
Standard error of the mean	1.8195	2.0423	5.6488	2.6072	3.0653	5.2194	3.2283	3.3631	3.7386

Statistics
One Way Analysis of Variance

Normality Test: Passed (P > 0.200)

Equal Variance Test: Passed (P = 0.014)

Group Name	N	Missing	Mean	Std Dev	SEM
6 control	6	0	10.817	0.440	0.180
6 5 min	6	0	11.400	0.494	0.202
6 15 min	6	0	11.733	1.366	0.558
30 control	6	0	10.683	0.631	0.257
30 5 min	6	0	11.183	0.741	0.303
30 control	6	0	8.317	0.781	0.319
36 5 min	6	0	11.383	0.813	0.332
36 15 min	6	0	10.717	0.904	0.369

Source of Variation	DF	SS	MS	F	P
Between Groups	8	96.623	12.078	15.676	<0.001
Residual	45	34.672	0.770		
Total	53	131.295			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
30 15 min vs. 36 control	5.500	9	15.348	<0.001	Yes
30 15 min vs. 30 control	3.133	9	8.744	<0.001	Yes
30 15 min vs. 3615 min	3.100	9	8.651	<0.001	Yes
30 15 min vs. 6 control	3.000	9	8.372	<0.001	Yes
30 15 min vs. 30 5 min	2.633	9	7.349	<0.001	Yes
30 15 min vs. 36 5 min	2.433	9	6.790	<0.001	Yes
30 15 min vs. 6 5 min	2.417	9	6.744	<0.001	Yes
30 15 min vs. 6 15 min	2.083	9	5.814	0.005	Yes
6 15 min vs. 36 control	3.417	9	9.534	<0.001	Yes
6 15 min vs. 30 control	1.050	9	2.930	0.504	No
6 15 min vs. 36 15 min	1.017	9	2.837	0.547	Do Not Test
6 15 min vs. 6 control	0.917	9	2.558	0.676	Do Not Test
6 15 min vs. 30 5 min	0.550	9	1.535	0.974	Do Not Test
6 15 min vs. 36 5 min	0.350	9	0.977	0.999	Do Not Test
6 15 min vs. 6 5 min	0.333	9	0.930	0.999	Do Not Test
6 5 min vs. 36 control	3.083	9	8.604	<0.001	Yes
6 5 min vs. 30 control	0.717	9	2.000	0.886	Do Not Test
6 5 min vs. 36 15 min	0.683	9	1.907	0.911	Do Not Test
6 5 min vs. 6 control	0.583	9	1.628	0.963	Do Not Test
6 5 min vs. 30 5 min	0.217	9	0.605	1.000	Do Not Test
6 5 min vs. 36 5 min	0.0167	9	0.0465	1.000	Do Not Test
36 5 min vs. 36 control	3.067	9	8.558	<0.001	Yes
36 5 min vs. 30 control	0.700	9	1.953	0.899	Do Not Test

4E-BP1 EDL

Film properties report 4E-BP1 EDL

Experimenter: Sreevani Uddemarri

Muscle : EDL Species: F344 X BN hybrid rat

Protein concentration: 60 µg/ml Gel type: 10% Tris-HCL SDSPAGE

Electrophoresis Voltage: 125V Transfer Voltage: 24V Duration: 45 min

Primary Antibody: 4E-BP1(Cell Signaling) Primary Antibody Dilution: 1:1000

Incubation Time: overnight @ 4°C Medium: 5% BSA

Secondary Antibody: Anti Rabbit Secondary Antibody Dilution: 1:1000

Incubation Time: 1hr @ room temp Medium: 5% milk in TBS-T

Exposure Time 3 min Molecular weight: 14.5kDa

Lane 1: Rainbow Marker RPN756 3µl

Lane 2: 6 month control

Lane 3: 6 month 5 min

Lane 4: 6 month 15 min

Lane 5: 30 month control

Lane 6: 30 month 5 min

Lane 7: 30 month 15min

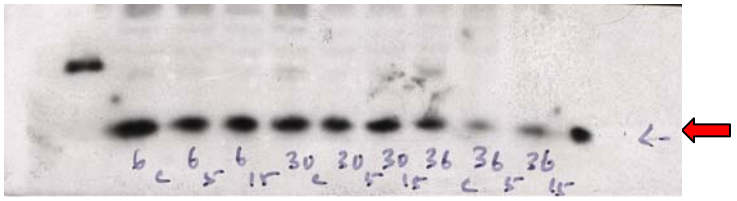
Lane 8: 36 month control

Lane 9: 36 month 5 min

Lane 10: 36 month 15 min

Lane 11: NIH 3T3 Activated Cell Extract 3µl

Lane 12: Biotinylated Ladder 3 µl



Raw data

This section represents the raw data tables Produced from spot densitometry of the immunoblot films.

4E-BP1 in EDL Data set

Raw IOD values (relative percentage)

	6 month	30 month	36 month
Raw values	11.6	14	9.7
	11.2	14	10.1
	11.6	13.8	10.2
	18.9	12.5	9.6
	18.8	12.2	9.1
	18.9	12.7	9.6
N	6	6	6
Mean	14.42	13.3	9.74
Strandard Deviation	6.910982	5.486134	3.995706
Standard Error of the mean	3.090685	2.453474	1.786934
% Relative expression	100	92.233	67.5451
SE	21.4333	17.0144	12.3921

Statistics
One Way Analysis of Variance

Normality Test: Passed (P = 0.049)
Equal Variance Test: Failed (P = <0.001)
Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
6 control	6	0	15.200	11.600	18.900
30 control	6	0	13.250	12.500	14.000
36 control	6	0	9.650	9.600	10.100

H = 11.416 with 2 degrees of freedom. (P = 0.003)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.003)

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0.05
6 control vs 36 control	54.000	4.129	Yes
6 control vs 30 control	0.000	0.000	No
30 control vs 36 control	54.00	4.129	Yes

p-4E-BP1 EDL

Film properties report p-4E-BP1 EDL

Experimenter: Sreevani Uddemarri

Muscle : EDL Species: F344 X BN hybrid rat

Protein concentration: 60 µg/ml Gel type: 10% Tris-HCL SDSPAGE

Electrophoresis Voltage: 125V Transfer Voltage: 24V Duration: 45 min

Primary Antibody: p-4E-BP1 (Cell Signaling) Primary Antibody Dilution: 1:1000

Incubation Time: overnight @ 4°C Medium: 5% BSA

Secondary Antibody: Anti Rabbit Secondary Antibody Dilution: 1:1000

Incubation Time: 1hr @ room temp Medium: 5% milk in TBS-T

Exposure Time 3 min Molecular weight: 14.5 kDa

Lane 1: Rainbow Marker RPN756 3µl

Lane 2: 6 month control

Lane 3: 6 month 5 min

Lane 4: 6 month 15 min

Lane 5: 30 month control

Lane 6: 30 month 5 min

Lane 7: 30 month 15min

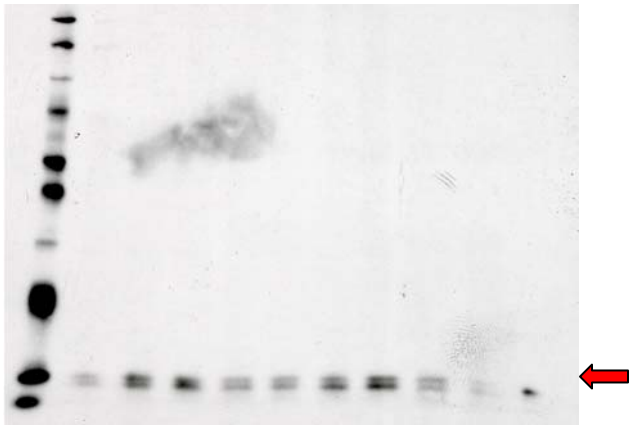
Lane 8: 36 month control

Lane 9: 36 month 5 min

Lane 10: 36 month 15 min

Lane 11: NIH 3T3 Activated Cell Extract 3µl

Lane 12: Biotinylated Ladder 3 µl



Raw data

This section represents the raw data tables Produced from spot densitometry of the immunoblot films.

p-4E-BP1 in EDL Data set

Raw IOD values (relative percentage)

	6 Control	**6 5 min**	**6 15 min**	**30 Control**	**30 5 min**	**30 15 min**	**36 Control**	**36 5 min**	**36 15 min**
Raw values	6.5	15.2	16.1	9	8.5	13.2	18.3	9	4.2
	6.3	14.9	15.2	8.9	8.6	13.5	18.1	9.7	4.9
	6.3	15.3	15.6	9	9	13.5	18	9	4.5
	9.8	12.1	16.3	11.2	13.3	9.8	10.9	11.8	4.7
	9.5	11.8	15.9	10.7	13	9.2	10.6	13.1	6.2
	9.9	12.3	16.9	11.5	15	9.5	11.2	9.8	4
N	6	6	6	6	6	6	6	6	6
Mean	8.05	13.6	16	10.05	11.23333	11.45	14.51667	10.4	4.75
Strandard Deviation	1.850135	1.692336	0.586515	1.214496	2.862633	2.147324	3.967577	1.674515	0.781665
Standard Error of the mean	0.827406	0.756836	0.262298	0.543139	1.280208	0.960312	1.774354	0.748866	0.349571
Relative Expression Level	100	168.9441	198.7578	124.8447	139.5445	142.236	180.3313	129.1925	59.0062
Standard error of the mean	10.2783	9.4017	3.2584	6.7471	15.9032	11.9293	22.0417	9.3027	4.3425

Statistics
One Way Analysis of Variance

Normality Test: Passed (P > 0.200)
Equal Variance Test: Failed (P = <0.001)
Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
6 control	6	0	8.000	6.300	9.800
6 5 min	6	0	13.600	12.100	15.200
6 15 min	6	0	16.000	15.600	16.300
30 control	6	0	9.850	9.000	11.200
30 5 min	6	0	11.000	8.600	13.300
30 15min	6	0	11.500	9.500	13.500
36 control	6	0	14.600	10.900	18.100
36 5 min	6	0	9.750	9.000	11.800
36 15 min	6	0	4.600	4.200	4.900

H = 36.715 with 8 degrees of freedom. (P = <0.001)
The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

4E-BP1 Soleus

Film properties report 4E-BP1 Soleus

Experimenter: Sreevani Uddemarri

Muscle : Soleus Species: F344 X BN hybrid rat

Protein concentration: 60 µg/ml Gel type: 10% Tris-HCL SDSPAGE

Electrophoresis Voltage: 125V Transfer Voltage: 24V Duration: 45 min

Primary Antibody: 4E-BP1(Cell Signaling) Primary Antibody Dilution: 1:1000

Incubation Time: overnight @ 4°C Medium: 5% BSA

Secondary Antibody: Anti Rabbit Secondary Antibody Dilution: 1:1000

Incubation Time: 1hr @ room temp Medium: 5% milk in TBS-T

Exposure Time 3 min Molecular weight: 14.5 kDa

Lane 1: Rainbow Marker RPN756 3µl

Lane 2: 6 month control

Lane 3: 6 month 5 min

Lane 4: 6 month 15 min

Lane 5: 30 month control

Lane 6: 30 month 5 min

Lane 7: 30 month 15min

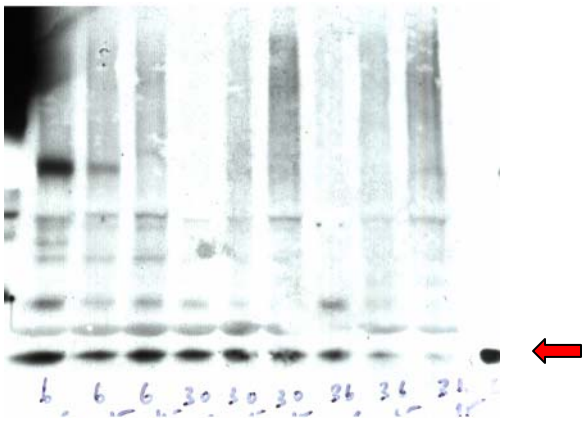
Lane 8: 36 month control

Lane 9: 36 month 5 min

Lane 10: 36 month 15 min

Lane 11: NIH 3T3 Activated Cell Extract 3µl

Lane 12: Biotinylated Ladder 3 µl



Raw data

This section represents the raw data tables Produced from spot densitometry of the immunoblot films.

4E-BP1 in Soleus Data set

Raw IOD values (relative percentage)

	6 month	30 month	36 month
Raw values	21.7	12.6	8.8
	21.8	12.6	8.7
	21.6	12.8	8.6
	30	8.3	11.3
	27.7	8.5	11.5
	27.4	8.4	11.2
N	6	6	6
Mean	25.03333	10.53333	10.01667
Strandard Deviation	3.761205	2.338946	1.446951
Standard Error of the mean	1.682062	1.046008	0.647096
% Relative expression	100	42.0772	40.0133
SE	6.7193	4.1785	2.5849

Statistics

One Way Analysis of Variance

Normality Test: Passed (P = 0.154)

Equal Variance Test: Failed (P = <0.001)

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
6control	6	0	24.600	21.700	27.700
30control	6	0	10.550	8.400	12.600
36control	6	0	10.000	8.700	11.300

H = 11.380 with 2 degrees of freedom. (P = 0.003)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.003)

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0.05
6 control vs 36 control	54.000	4.129	Yes
6 control vs 30 control	54.000	4.12	Yes
30 control vs 36 control	0.000	0.000	No

p-4E-BP1 Soleus

Film properties report p-4E-BP1 Soleus

Experimenter: Sreevani Uddemarri

Muscle : Soleus Species: F344 X BN hybrid rat

Protein concentration: 60 µg/ml Gel type: 7.5% Tris-HCL SDSPAGE

Electrophoresis Voltage: 125V Transfer Voltage: 24V Duration: 45 min

Primary Antibody: p-4E-BP1 (Cell Signaling) Primary Antibody Dilution: 1:1000

Incubation Time: overnight @ 4°C Medium: 5% BSA

Secondary Antibody: Anti Rabbit Secondary Antibody Dilution: 1:1000

Incubation Time: 1hr @ room temp Medium: 5% milk in TBS-T

Exposure Time 3 min Molecular weight: 14.5 kDa

Lane 1: Rainbow Marker RPN756 3µl

Lane 2: 6 month control

Lane 3: 6 month 5 min

Lane 4: 6 month 15 min

Lane 5: 30 month control

Lane 6: 30 month 5 min

Lane 7: 30 month 15min

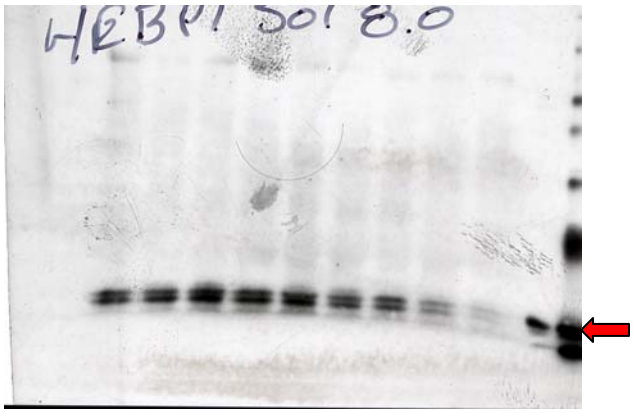
Lane 8: 36 month control

Lane 9: 36 month 5 min

Lane 10: 36 month 15 min

Lane 11: NIH 3T3 Activated Cell Extract 3µl

Lane 12: Biotinylated Ladder 3 µl



Raw data

This section represents the raw data tables Produced from spot densitometry of the immunoblot films.

p-4E-BP1 in Soleus Data set

Raw IOD values (relative percentage)

	6 Control	**6 5 min**	**6 15 min**	**30 Control**	**30 5 min**	**30 15 min**	**36 Control**	**36 5 min**	**36 15 min**
Raw values	11.4	12.5	16	14.7	14.1	10.3	9.5	6.5	5.1
	11.9	12.4	16.1	14	14.4	10.3	10	6.7	4.3
	11.7	12.2	16.1	14.1	13.7	10.4	10.5	7.3	4.1
	11	13.8	17.3	12.7	10.7	13.3	11.5	6.1	3.7
	11	14.4	17	12.7	10.5	12.7	11.9	5.9	3.7
	10.7	13.8	17.3	12.7	10.4	13	12.1	6.3	3.7
N	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**11.28333**	**13.18333**	**16.63333**	**13.48333**	**12.3**	**11.66667**	**10.91667**	**6.466667**	**4.1**
Strandard Deviation	0.462241	0.926103	0.631401	0.89088	1.950385	1.473318	1.070358	0.496655	0.551362
Standard Error of the mean	**0.20672**	**0.414166**	**0.282371**	**0.398414**	**0.872238**	**0.658888**	**0.478679**	**0.222111**	**0.246577**
Relative Expression Level	**100**	**116.839**	**147.4151**	**119.4978**	**109.0103**	**103.3973**	**96.7504**	**57.3117**	**36.3368**
Standard error of the mean	**1.8321**	**3.6706**	**2.5025**	**3.531**	**7.7303**	**5.8395**	**4.2424**	**1.9685**	**2.1853**

Statistics

One Way Analysis of Variance
Normality Test: Passed (P > 0.200)
Equal Variance Test: Failed (P = <0.001)
Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
6 control	6	0	11.200	11.000	11.700
6 5 min	6	0	13.150	12.400	13.800
6 15 min	6	0	16.550	16.100	17.300
30 control	6	0	13.350	12.700	14.100
30 5 min	6	0	12.200	10.500	14.100
30 15 min	6	0	11.550	10.300	13.000
36 control	6	0	11.000	10.000	11.900
36 5 min	6	0	6.400	6.100	6.700
36 15 min	6	0	3.900	3.700	4.300

H = 43.647 with 8 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0.05
615 min vs 3615 min	288.000	7.474	Yes
615 min vs 365 min	252.000	6.539	Yes
615 min vs 36 control	178.000	4.619	Yes
615 min vs 6 control	160.000	4.152	No
615 min vs 3015 min	148.000	3.841	Do Not Test
615 min vs 305 min	119.500	3.101	Do Not Test
615 min vs 65 min	83.500	2.167	Do Not Test
615 min vs 30 control	67.000	1.739	Do Not Test
30 control vs 36 15 min	221.000	5.735	Yes
30control vs 365 min	185.000	4.801	Yes
30control vs 36control	111.000	2.880	No
30control vs 6 control	93.000	2.413	Do Not Test
30control vs 3015 min	81.000	2.102	Do Not Test
30control vs 305 min	52.500	1.362	Do Not Test
30 control vs 65 min	16.500	0.428	Do Not Test
65 min vs 3615 min	204.500	5.307	Yes
65 min vs 365 min	168.500	4.373	No
65 min vs 36 control	94.500	2.452	Do Not Test
65 min vs 6 control	76.500	1.985	Do Not Test
65 min vs 3015 min	64.500	1.674	Do Not Test

mTOR EDL

Film properties report mTOR EDL

Experimenter: Sreevani Uddemarri

Muscle : EDL Species: F344 X BN hybrid rat

Protein concentration: 60 µg/ml Gel type: 7.5% Tris-HCL SDSPAGE

Electrophoresis Voltage: 125V Transfer Voltage: 24V Duration: 45 min

Primary Antibody: mTOR (Cell Signaling) Primary Antibody Dilution: 1:1000

Incubation Time: overnight @ 4°C Medium: 5% BSA

Secondary Antibody: Anti Rabbit Secondary Antibody Dilution: 1:1000

Incubation Time: 1hr @ room temp Medium: 5% milk in TBS-T

Exposure Time 3 min Molecular weight: 280 kDa

Lane 1: Rainbow Marker RPN756 3µl

Lane 2: 6 month control

Lane 3: 6 month 5 min

Lane 4: 6 month 15 min

Lane 5: 30 month control

Lane 6: 30 month 5 min

Lane 7: 30 month 15min

Lane 8: 36 month control

Lane 9: 36 month 5 min

Lane 10: 36 month 15 min

Lane 11: NIH 3T3 Activated Cell Extract 3µl

Lane 12: Biotinylated Ladder 3 µl

Raw data

This section represents the raw data tables Produced from spot densitometry of the immunoblot films.

mTOR in EDL Data set

Raw IOD values (relative percentage)

	6 month	30 month	36 month
Raw values	47.3	25.1	27.6
	53.7	22	24.2
	40.3	31.1	28.6
	41.2	30.1	28.7
	42.1	26.7	31.2
	40.4	27.5	32
N	**6**	**6**	**6**
Mean	**44.16667**	**27.08333**	**28.71667**
Strandard Deviation	5.346276	3.32651	2.778789
Standard Error of the mean	**2.390927**	**1.48766**	**1.242712**
% Relative expression	100	61.3208	65.0189
SE	5.4134	3.3683	2.8137

Statistics
One Way Analysis of Variance
Normality Test: Passed (P > 0.200)
Equal Variance Test: Passed (P = 0.658)

Group Name	N	Missing	Mean	Std Dev	SEM
6 control	6	0	44.167	5.346	2.183
30 control	6	0	27.083	3.327	1.358
36 control	6	0	28.717	2.779	1.134

Source of Variation	DF	SS	MS	F	P
Between Groups	2	1066.421	533.211	33.769	<0.001
Residual	15	236.850	15.790		
Total	17	1303.271			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
6 control vs. 30 control	17.083	3	10.531	<0.001	Yes
6 control vs. 36 control	15.450	3	9.524	<0.001	Yes
36 control vs. 30 control	1.633	3	1.007	0.760	No

p-mTOR EDL

Film properties report p-mTOR EDL

Experimenter: Sreevani Uddemarri Muscle : EDL Species: F344 X BN hybrid rat

Protein concentration: 60 µg/ml Gel type: 7.5% Tris-HCL SDSPAGE

Electrophoresis Voltage: 125V Transfer Voltage: 24V Duration: 45 min

Primary Antibody: p-mTOR (Cell Signaling) Primary Antibody Dilution: 1:1000

Incubation Time: overnight @ 4°C Medium: 5% BSA

Secondary Antibody: Anti Rabbit Secondary Antibody Dilution: 1:1000

Incubation Time: 1hr @ room temp Medium: 5% milk in TBS-T

Exposure Time 3 min Molecular weight: 42, 44 kDa

Lane 1: Rainbow Marker RPN756 3µl

Lane 2: 6 month control

Lane 3: 6 month 5 min

Lane 4: 6 month 15 min

Lane 5: 30 month control

Lane 6: 30 month 5 min

Lane 7: 30 month 15min

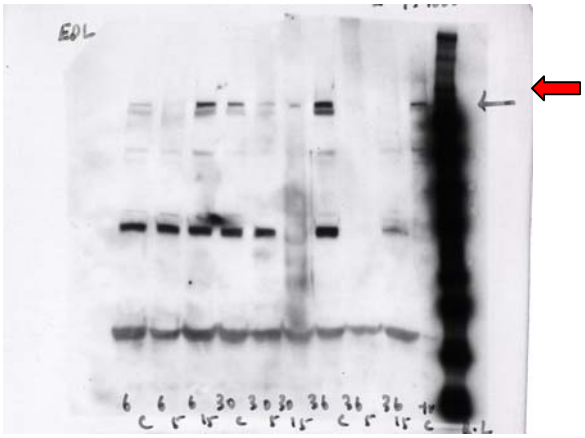
Lane 8: 36 month control

Lane 9: 36 month 5 min

Lane 10: 36 month 15 min

Lane 11: NIH 3T3 Activated Cell Extract 3µl

Lane 12: Biotinylated Ladder 3 µl



Raw data

This section represents the raw data tables Produced from spot densitometry of the immunoblot films.

p-mTOR in EDL Data set

Raw IOD values (relative percentage)

	6 Control	**6 5 min**	**6 15 min**	**30 Control**	**30 5 min**	**30 15 min**	**36 Control**	**36 5 min**	**36 15 min**
Raw values	9	8.4	19.2	10.1	8.8	8	24.9	5.8	5.8
	9.5	7.1	20.3	10.8	8.9	7.2	26.8	4.8	4.6
	10.1	9	15.4	9.5	13.1	12.1	19.1	7.6	4.1
	10.6	11.8	15.4	7.8	12.2	12.7	19.1	6.6	3.6
	4.7	5.9	13.1	12.3	8.5	5.5	32.2	8.9	8.8
	4.3	5.2	13.4	12.6	8.2	5.6	31.7	9.5	9.4
N	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**8.033333**	**7.9**	**16.13333**	**10.51667**	**9.95**	**8.516667**	**25.63333**	**7.2**	**6.05**
Strandard Deviation	2.792609	2.391652	2.983734	1.799352	2.124853	3.160643	5.782618	1.812181	2.480121
Standard Error of the mean	**1.248893**	**1.069579**	**1.334366**	**0.804695**	**0.950263**	**1.413483**	**2.586065**	**0.810432**	**1.109144**
Relative Expression Level	100	98.34025	200.8299	130.9129	123.8589	106.0166	319.0871	89.62656	75.3112
Standard error of the mean	15.54638	13.31427	16.61037	10.01694	11.829	17.59522	32.19168	10.08836	13.80677

Statistics

One way analysis of variance

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
36 control vs. 3615 min	19.583	9	15.792	<0.001	Yes
36 control vs. 36 5 min	18.433	9	14.865	<0.001	Yes
36 control vs. 6 5 min	17.733	9	14.300	<0.001	Yes
36 control vs. 6 control	17.600	9	14.193	<0.001	Yes
36 control vs. 30 15 min	17.117	9	13.803	<0.001	Yes
36 control vs. 30 5 min	15.683	9	12.647	<0.001	Yes
36 control vs. 30 control	15.117	9	12.190	<0.001	Yes
36 control vs. 6 15 min	9.500	9	7.661	<0.001	Yes
6 15 min vs. 3615 min	10.083	9	8.131	<0.001	Yes
6 15 min vs. 36 5 min	8.933	9	7.204	<0.001	Yes
6 15 min vs. 6 5 min	8.233	9	6.640	<0.001	Yes
6 15 min vs. 6 control	8.100	9	6.532	0.001	Yes
6 15 min vs. 3015 min	7.617	9	6.142	0.002	Yes
6 15 min vs. 30 5 min	6.183	9	4.986	0.025	Yes
6 15 min vs. 30 control	5.617	9	4.529	0.057	No
30 control vs. 3615 min	4.467	9	3.602	0.238	No
30 control vs. 365 min	3.317	9	2.675	0.623	Do Not Test
30 control vs. 6 5 min	2.617	9	2.110	0.853	Do Not Test
30 control vs. 6 control	2.483	9	2.003	0.886	Do Not Test
30 control vs. 30 15 min	2.000	9	1.613	0.964	Do Not Test
30 control vs. 30 5 min	0.567	9	0.457	1.000	Do Not Test
30 5 min vs. 36 15 min	3.900	9	3.145	0.409	Do Not Test
30 5 min vs. 36 5 min	2.750	9	2.218	0.816	Do Not Test

mTOR Soleus

Film properties report mTOR Soleus

Experimenter: Sreevani Uddemarri

Muscle : Soleus Species: F344 X BN hybrid rat

Protein concentration: 60 µg/ml Gel type: 7.5% Tris-HCL SDSPAGE

Electrophoresis Voltage: 125V Transfer Voltage: 24V Duration: 45 min

Primary Antibody: mTOR (Cell Signaling) Primary Antibody Dilution: 1:1000

Incubation Time: overnight @ 4°C Medium: 5% BSA

Secondary Antibody: Anti Rabbit Secondary Antibody Dilution: 1:1000

Incubation Time: 1hr @ room temp Medium: 5% milk in TBS-T

Exposure Time 3 min Molecular weight: 280 kDa

Lane 1: Rainbow Marker RPN756 3µl

Lane 2: 6 month control

Lane 3: 6 month 5 min

Lane 4: 6 month 15 min

Lane 5: 30 month control

Lane 6: 30 month 5 min

Lane 7: 30 month 15min

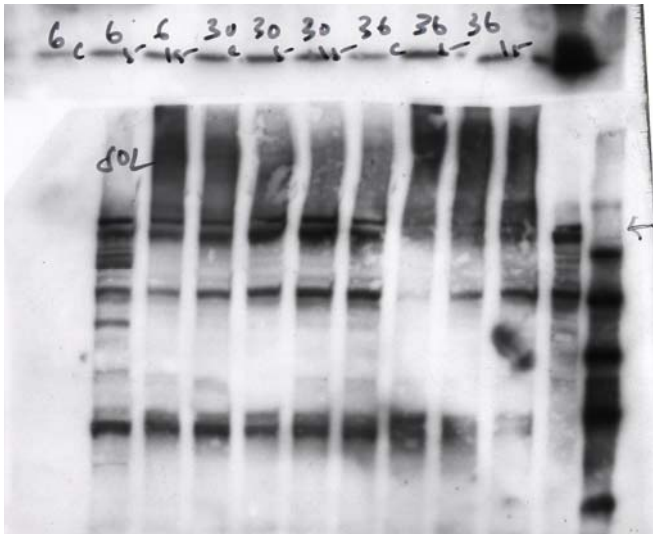
Lane 8: 36 month control

Lane 9: 36 month 5 min

Lane 10: 36 month 15 min

Lane 11: NIH 3T3 Activated Cell Extract 3µl

Lane 12: Biotinylated Ladder 3 µl



Raw data

This section represents the raw data tables Produced from spot densitometry of the immunoblot films.

mTOR in Soleus Data set

Raw IOD values (relative percentage)

	6 month	30 month	36 month
Raw values	40.4	39.1	20.5
	39.9	39.9	20.3
	40.2	37.4	22.4
	40.1	37.8	22.1
	40.4	40.8	18.8
	39.1	39.7	21.1
N	6	6	6
Mean	40.01667	39.11667	20.86667
Strandard Deviation	0.487511	1.301409	1.315548
Standard Error of the mean	0.218021	0.582008	0.588331
% Relative expression	100	97.7509	52.1449
SE	0.5448	1.4544	1.4702

Statistics
One Way Analysis of Variance
Normality Test: Passed (P > 0.200)
Equal Variance Test: Passed (P = 0.130)

Group Name	N	Missing	Mean	Std Dev	SEM
6 control	6	0	40.017	0.488	0.199
30 control	6	0	39.117	1.301	0.531
36 control	6	0	20.867	1.316	0.537

Source of Variation	DF	SS	MS	F	P
Between Groups	2	1401.190	700.595	573.945	<0.001
Residual	15	18.310	1.221		
Total	17	1419.500			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).
Power of performed test with alpha = 0.050: 1.000

p-mTOR Soleus

Film properties report p-mTOR Soleus

Experimenter: Sreevani Uddemarri

Muscle : Soleus Species: F344 X BN hybrid rat

Protein concentration: 60 µg/ml Gel type: 7.5% Tris-HCL SDSPAGE

Electrophoresis Voltage: 125V Transfer Voltage: 24V Duration: 45 min

Primary Antibody: p-mTOR (Cell Signaling) Primary Antibody Dilution: 1:1000

Incubation Time: overnight @ 4°C Medium: 5% BSA

Secondary Antibody: Anti Rabbit Secondary Antibody Dilution: 1:1000

Incubation Time: 1hr @ room temp Medium: 5% milk in TBS-T

Exposure Time 3 min Molecular weight: 42, 44 kDa

Lane 1: Rainbow Marker RPN756 3µl

Lane 2: 6 month control

Lane 3: 6 month 5 min

Lane 4: 6 month 15 min

Lane 5: 30 month control

Lane 6: 30 month 5 min

Lane 7: 30 month 15min

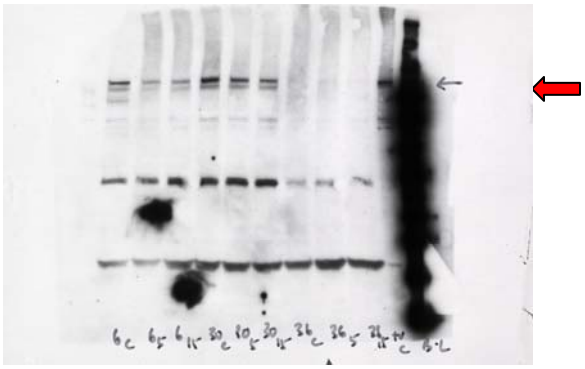
Lane 8: 36 month control

Lane 9: 36 month 5 min

Lane 10: 36 month 15 min

Lane 11: NIH 3T3 Activated Cell Extract 3µl

Lane 12: Biotinylated Ladder 3 µl



Raw data

This section represents the raw data tables Produced from spot densitometry of the immunoblot films.

p-mTOR in SoleusData set

Raw IOD values (relative percentage)

	6 Control	**6 5 min**	**6 15 min**	**30 Control**	**30 5 min**	**30 15 min**	**36 Control**	**36 5 min**	**36 15 min**
Raw values	12.4	10.6	10.6	18.4	15.7	13.4	9.4	5.8	3.6
	13.1	10.2	10.5	17.6	14.9	13.7	9.3	6.3	4.4
	15.1	10.3	11.1	19.2	14.6	13.1	8.2	5	3.3
	14.8	14.8	11	18.5	10.6	9.2	6.5	7.8	6.8
	14.3	16.6	10.9	19.1	10.5	8.8	6.1	7.3	6.3
	13.2	16.4	11.4	17.8	10.9	8.8	6.7	7.2	7.5
N	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**13.81667**	**13.15**	**10.91667**	**18.43333**	**12.86667**	**11.16667**	**7.7**	**6.566667**	**5.316667**
Strandard Deviation	1.072225	3.114964	0.33116	0.653197	2.440219	2.458184	1.462874	1.055778	1.776982
Standard Error of the mean	**0.479514**	**1.393054**	**0.148099**	**0.292119**	**1.091299**	**1.099333**	**0.654217**	**0.472158**	**0.794691**
Relative Expression Level	**100**	**95.17491**	**79.01086**	**133.4138**	**93.12425**	**80.82027**	**55.72979**	**47.52714**	**38.4801**
Standard error of the mean	**3.470545**	**10.08242**	**1.071887**	**2.114249**	**7.898424**	**7.956573**	**4.734985**	**3.417309**	**5.751682**

Statistics

One Way Analysis of Variance

Normality Test: Passed (P > 0.200)
Equal Variance Test: Passed (P = 0.649)

Group Name	N	Missing	Mean	Std Dev	SEM
6 control	6	0	14.867	1.763	0.720
6 5 min	6	0	12.233	3.058	1.248
6 15 min	6	0	10.533	0.944	0.385
30 control	6	0	16.117	2.766	1.129
30 5 min	6	0	11.133	2.606	1.064
30 15 min	6	0	10.183	2.375	0.970
36 control	6	0	8.683	1.659	0.677
36 5 min	6	0	8.300	2.770	1.131
3615 min	6	0	7.983	3.451	1.409

Source of Variation	DF	SS	MS	F	P
Between Groups	8	391.161	48.895	7.891	<0.001
Residual	45	278.827	6.196		
Total	53	669.988			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
30 control vs. 3615 min	8.133	9	8.004	<0.001	Yes
30 control vs. 365 min	7.817	9	7.692	<0.001	Yes
30 control vs. 36 control	7.433	9	7.315	<0.001	Yes
30 control vs. 3015 min	5.933	9	5.839	0.005	Yes
30 control vs. 615 min	5.583	9	5.494	0.009	Yes
30 control vs. 305 min	4.983	9	4.904	0.029	Yes
30 control vs. 65 min	3.883	9	3.821	0.176	No
30 control vs. 6 control	1.250	9	1.230	0.994	Do Not Test
6 control vs. 3615 min	6.883	9	6.774	<0.001	Yes
6 control vs. 365 min	6.567	9	6.462	0.001	Yes
6 control vs. 36 control	6.183	9	6.085	0.003	Yes
6 control vs. 3015 min	4.683	9	4.609	0.050	Yes
6 control vs. 615 min	4.333	9	4.264	0.089	No
6 control vs. 305 min	3.733	9	3.674	0.216	Do Not Test
6 control vs. 65 min	2.633	9	2.591	0.661	Do Not Test
65 min vs. 3615 min	4.250	9	4.182	0.102	No
65 min vs. 365 min	3.933	9	3.871	0.164	Do Not Test
65 min vs. 36 control	3.550	9	3.493	0.274	Do Not Test
65 min vs. 3015 min	2.050	9	2.017	0.882	Do Not Test
65 min vs. 615 min	1.700	9	1.673	0.956	Do Not Test
65 min vs. 305 min	1.100	9	1.082	0.997	Do Not Test
305 min vs. 3615 min	3.150	9	3.100	0.428	Do Not Test
305 min vs. 365 min	2.833	9	2.788	0.570	Do Not Test

GSK-3ß EDL

Film properties report GSK-3ß EDL

Experimenter: Sreevani Uddemarri

Muscle : EDL Species: F344 X BN hybrid rat

Protein concentration: 60 µg/ml Gel type: 10% Tris-HCL SDSPAGE

Electrophoresis Voltage: 125V Transfer Voltage: 24V Duration: 45 min

Primary Antibody: GSK-3ß(Cell Signaling) Primary Antibody Dilution: 1:1000

Incubation Time: overnight @ 4°C Medium: 5% BSA

Secondary Antibody: Anti Rabbit Secondary Antibody Dilution: 1:1000

Incubation Time: 1hr @ room temp Medium: 5% milk in TBS-T

Exposure Time 3 min Molecular weight: 45 kDa

Lane 1: Rainbow Marker RPN756 3µl

Lane 2: 6 month control

Lane 3: 6 month 5 min

Lane 4: 6 month 15 min

Lane 5: 30 month control

Lane 6: 30 month 5 min

Lane 7: 30 month 15min

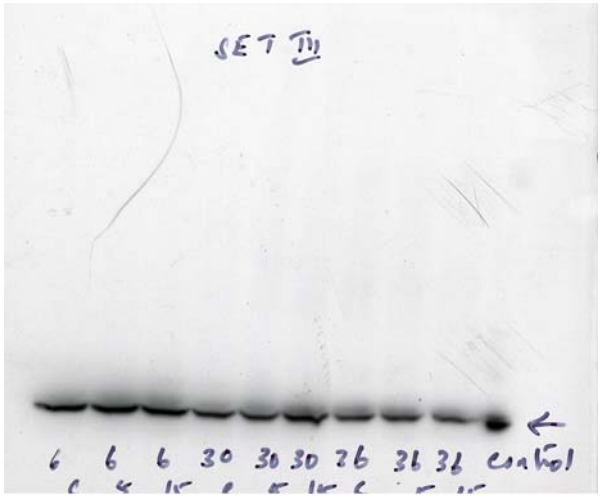
Lane 8: 36 month control

Lane 9: 36 month 5 min

Lane 10: 36 month 15 min

Lane 11: NIH 3T3 Activated Cell Extract 3µl

Lane 12: Biotinylated Ladder 3 µl



Raw data

This section represents the raw data tables Produced from spot densitometry of the immunoblot films.

GSK-3ß in EDL Data set

Raw IOD values (relative percentage)

	6 month	30 month	36 month
Raw values	13.8	16.1	7.3
	13.4	13.5	7.4
	15.9	12.6	8.4
	12.3	15.8	5.1
	10.8	17.6	8.3
	15.9	13.9	8
	14.6	13.7	8.3
	12.6	13.8	8
N	8	8	8
Mean	13.6625	14.625	7.6
Strandard Deviation	1.779195	1.683322	1.090216
Standard Error of the mean	0.672473	0.636236	0.412063
% Relative expression	100	107.0448	55.6267
SE	4.922	4.6568	3.016

Statistics

One Way Analysis of Variance

Normality Test: Passed (P > 0.200)
Equal Variance Test: Passed (P = 0.390)

Group Name	N	Missing	Mean	Std Dev	SEM
6 months	8	0	13.663	1.779	0.629
30 months	8	0	14.62	1.683	0.595
36 months	8	0	7.60	1.09	0.385

Source of Variation	DF	SS	MS	F	P
Between Groups	2	232.082	116.041	48.433	<0.001
Residual	21	50.314	2.396		
Total	23	282.396			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000
All Pairwise Multiple Comparison Procedures (Tukey Test):

:

Comparison	Diff of Means	p	q	P	P<0.050
30 months vs. 36 months	7.025	3	12.837	<0.001	Yes
30 months vs. 6 months	0.963	3	1.759	0.442	No
6 months vs. 36 months	6.063	3	11.078	<0.001	Yes

p-GSK-3ß EDL

Film properties report p-GSK-3ß EDL

Experimenter: Sreevani Uddemarri Muscle : EDL Species: F344 X BN hybrid rat

Protein concentration: 60 µg/ml Gel type: 10% Tris-HCL SDSPAGE

Electrophoresis Voltage: 125V Transfer Voltage: 24V Duration: 45 min

Primary Antibody: p- GSK-3ß(Cell Signaling) Primary Antibody Dilution: 1:1000

Incubation Time: overnight @ 4°C Medium: 5% BSA

Secondary Antibody: Anti Rabbit Secondary Antibody Dilution: 1:1000

Incubation Time: 1hr @ room temp Medium: 5% milk in TBS-T

Exposure Time 5 sec Molecular weight: 45 kDa

Lane 1: Rainbow Marker RPN756 3µl

Lane 2: 6 month control

Lane 3: 6 month 5 min

Lane 4: 6 month 15 min

Lane 5: 30 month control

Lane 6: 30 month 5 min

Lane 7: 30 month 15min

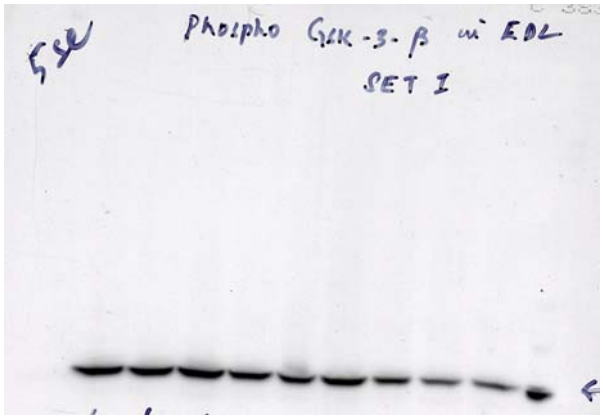
Lane 8: 36 month control

Lane 9: 36 month 5 min

Lane 10: 36 month 15 min

Lane 11: NIH 3T3 Activated Cell Extract 3µl

Lane 12: Biotinylated Ladder 3 µl



Raw data

This section represents the raw data tables Produced from spot densitometry of the immunoblot films.

p- GSK-3ß in EDL Data set

Raw %IOD values

Raw IOD values (relative percentage)

	6 Control	**6 5 min**	**6 15 min**	**30 Control**	**30 5 min**	**30 15 min**	**36 Control**	**36 5 min**	**36 15 min**
Raw values	14.3	13.6	14.8	11.2	10.3	12.4	8.8	7	7.5
	14.5	12.3	14.5	11.2	10.6	12.4	9.3	7.5	7.8
	15.2	12.8	14.6	11.9	10	11.9	8.7	7.2	7.8
	13.8	12	14.2	11.6	10.8	12.6	9.3	7	8.7
	13.9	12.1	14.4	10.6	10.7	11.8	10.6	7.4	8.3
	13.1	14.5	16	13.4	10.9	11.5	7.7	6.3	6.6
	13.6	14.5	15.8	12.7	10.8	11.2	7.9	6.7	6.9
	13.3	13.9	16.1	13.1	9.8	11.7	8.3	7.1	6.8
	14.3	13.6	14.8	11.2	10.3	12.4	8.8	7	7.5
N	8	8	8	8	8	8	8	8	8
Mean	13.9625	13.2125	15.05	11.9625	10.4875	11.9375	8.825	7.025	7.55
Strandard Deviation	0.684392	1.045313	0.781939	1.004188	0.408613	0.489716	0.926977	0.384522	0.74642
Standard Error of the mean	0.258676	0.395091	0.295545	0.379547	0.154441	0.185095	0.350364	0.145336	0.28212
Relative Expression Level	100	94.6285	107.7887	85.6759	75.1119	85.4969	63.205	50.3133	54.0734
Standard error of the mean	1.8526	2.8297	2.1167	2.7183	1.1061	1.3257	2.5093	1.0409	2.0206

Statistics
One Way Analysis of Variance
Normality Test: Passed (P > 0.200)
Equal Variance Test: Passed (P = 0.037)

Group Name	N	Missing	Mean	Std Dev	SEM
6 5	8	0	13.213	1.045	0.370
6 15	8	0	15.050	0.782	0.276
30 control	8	0	11.963	1.004	0.355
30 5	8	0	10.488	0.409	0.144
30 15	8	0	11.938	0.490	0.173
36 control	8	0	8.825	0.927	0.328
36 5	8	0	7.025	0.385	0.136
36 15	8	0	7.550	0.746	0.264

Source of Variation	DF	SS	MS	F	P
Between Groups	7	442.592	63.227	107.999	<0.001
Residual	56	32.785	0.585		
Total	63	475.377			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).
Power of performed test with alpha = 0.050: 1.000

GSK-3ß Soleus

Film properties report GSK-3ß Soleus

Experimenter: Sreevani Uddemarri

Muscle : Soleus Species: F344 X BN hybrid rat

Protein concentration: 60 µg/ml Gel type: 10% Tris-HCL SDSPAGE

Electrophoresis Voltage: 125V Transfer Voltage: 24V Duration: 45 min

Primary Antibody: GSK-3ß(Cell Signaling) Primary Antibody Dilution: 1:1000

Incubation Time: overnight @ 4°C Medium: 5% BSA

Secondary Antibody: Anti Rabbit Secondary Antibody Dilution: 1:1000

Incubation Time: 1hr @ room temp Medium: 5% milk in TBS-T

Exposure Time 3 min Molecular weight: 45kDa

Lane 1: Rainbow Marker RPN756 3µl

Lane 2: 6 month control

Lane 3: 6 month 5 min

Lane 4: 6 month 15 min

Lane 5: 30 month control

Lane 6: 30 month 5 min

Lane 7: 30 month 15min

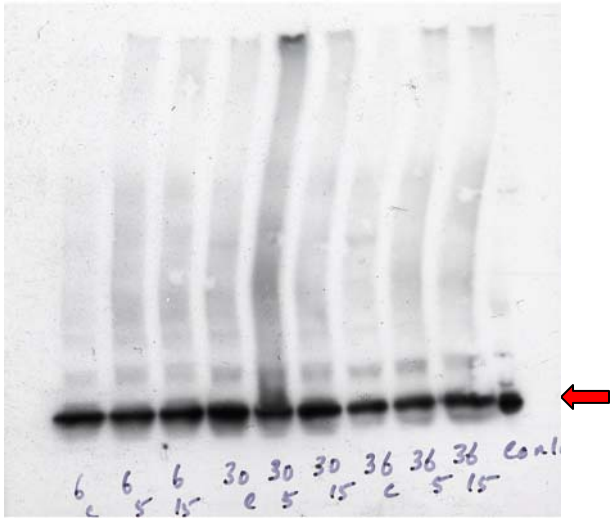
Lane 8: 36 month control

Lane 9: 36 month 5 min

Lane 10: 36 month 15 min

Lane 11: NIH 3T3 Activated Cell Extract 3µl

Lane 12: Biotinylated Ladder 3 µl



Raw data

This section represents the raw data tables Produced from spot densitometry of the immunoblot films.

GSK-3ß in Soleus Data set

Raw IOD values (relative percentage)

	6 months	**30 months**	**36 months**
Raw values	42.1	30.9	26.9
	38.7	35	26.3
	39.1	32.9	28.1
	42.9	28.2	28.9
	44.8	26.4	28.8
	38.4	32.8	28.8
	38.4	32.9	28.7
	39.6	33	27.4
	43.8	28.1	28.1
N	**9**	**9**	**9**
Mean	**40.86667**	**31.13333**	**28**
Strandard Deviation	2.532785	2.909467	0.939415
Standard Error of the mean	**0.895475**	**1.028652**	**0.332133**
% Relative expression	**100**	**76.1827**	**68.5155**
SE	**2.1912**	**2.5171**	**0.8127**

Statistics
One Way Analysis of Variance

Normality Test: Passed (P > 0.200)
Equal Variance Test: Passed (P = 0.193)

Group Name	N	Missing	Mean	Std Dev	SEM
6 month	9	0	40.867	2.533	0.844
30 month	9	0	31.133	2.909	0.970
36 month	9	0	28.000	0.939	0.313

Source of Variation	DF	SS	MS	F	P
Between Groups	2	810.320	405.160	77.112	<0.001
Residual	24	126.100	5.254		
Total	26	936.420			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
6 months vs. 36 months	12.867	3	16.840	<0.001	Yes
6 months vs. 30 months	9.733	3	12.739	<0.001	Yes
30 months vs. 36 months	3.133	3	4.101	0.021	Yes

p- GSK-3ß Soleus

Film properties report p- GSK-3ß Soleus

Experimenter: Sreevani Uddemarri

Muscle : Soleus Species: F344 X BN hybrid rat

Protein concentration: 60 µg/ml Gel type: 10% Tris-HCL SDSPAGE

Electrophoresis Voltage: 125V Transfer Voltage: 24V Duration: 45 min

Primary Antibody: p- GSK-3ß(Cell Signaling) Primary Antibody Dilution: 1:1000

Incubation Time: overnight @ 4°C Medium: 5% BSA

Secondary Antibody: Anti Rabbit Secondary Antibody Dilution: 1:1000

Incubation Time: 1hr @ room temp Medium: 5% milk in TBS-T

Exposure Time 15 sec Molecular weight: 45 kDa

Lane 1: Rainbow Marker RPN756 3µl

Lane 2: 6 month control

Lane 3: 6 month 5 min

Lane 4: 6 month 15 min

Lane 5: 30 month control

Lane 6: 30 month 5 min

Lane 7: 30 month 15min

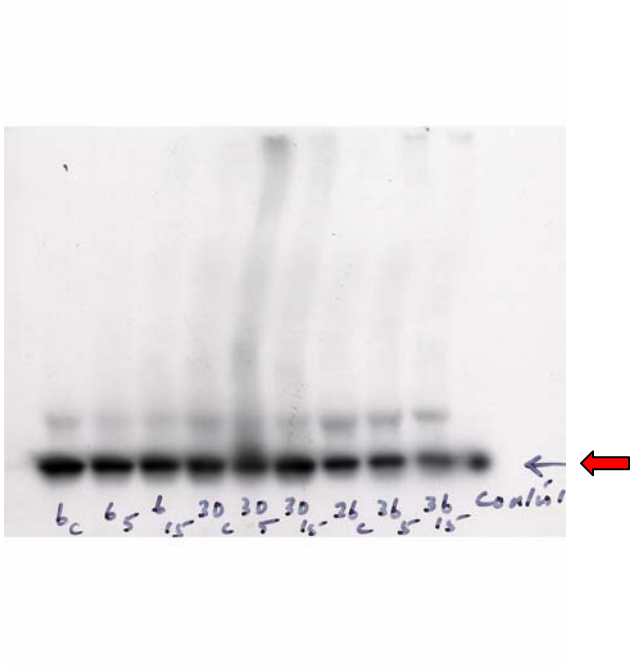
Lane 8: 36 month control

Lane 9: 36 month 5 min

Lane 10: 36 month 15 min

Lane 11: NIH 3T3 Activated Cell Extract 3µl

Lane 12: Biotinylated Ladder 3 µl



Raw data

This section represents the raw data tables Produced from spot densitometry of the immunoblot films.

p- GSK-3ß in Soleus Data set

Raw IOD values (relative percentage)

	6 Control	**6 5 min**	**6 15 min**	**30 Control**	**30 5 min**	**30 15 min**	**36 Control**	**36 5 min**	**36 15 min**
Raw values	15.7	11.2	11.8	10.8	8.5	11.7	10.4	9.4	10.5
	15.4	11.4	11.7	11.1	9.1	11.1	10.1	9.6	10.5
	16.2	11.1	11.3	11.4	9.2	11.2	10.2	9.2	10.2
	14.1	9.9	10.2	10.6	9.9	10.4	10.8	10.8	13.2
	14.8	10.8	10.4	11	10	10.3	10.6	9.8	12.4
	14.5	11.9	11.1	10.9	10.1	10.1	10.1	10.1	11.1
	15.6	12.1	11.3	10.4	9.5	12.8	10.5	9.7	8.2
	14.9	11.6	10.6	11	10.4	13.4	10.6	9.3	8.1
	15.7	12.5	11.3	9.4	10.1	13.6	11	8.7	7.7
N	9	9	9	9	9	9	9	9	9
Mean	15.21111	11.38889	11.07778	10.73333	9.644444	11.62222	10.47778	9.622222	10.21111
Strandard Deviation	0.67536	0.768838	0.560753	0.576628	0.612599	1.345156	0.311359	0.595352	1.919925
Standard Error of the mean	0.238776	0.271825	0.198256	0.203869	0.216587	0.475584	0.110082	0.210489	0.678796
Relative Expression Level	100	74.8722	72.8269	70.5625	63.4039	76.4061	68.8824	63.2579	67.1293
Standard error of the mean	1.5697	1.787	1.3034	1.3403	1.4239	3.1266	0.7237	1.3838	4.4625

Statistics
One Way Analysis of Variance
Normality Test: Failed (P = 0.008)
Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
6 control	9	0	15.400	14.725	15.700
65 min	9	0	11.400	11.025	11.950
615 min	9	0	11.300	10.550	11.400
30 control	9	0	10.900	10.550	11.025
30 5 min	9	0	9.900	9.175	10.100
3015 min	9	0	11.200	10.375	12.950
36 control	9	0	10.500	10.175	10.650
36 5 min	9	0	9.600	9.275	9.875
3615 min	9	0	10.500	8.175	11.425

H = 50.198 with 8 degrees of freedom. (P = <0.001)
The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)
To isolate the group or groups that differ from the others use a multiple comparison procedure.
All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0.05
6 control vs 30 5 min	555.000	7.863	Yes
6 control vs 365 min	555.000	7.863	Yes
6 control vs 3615 min	403.000	5.710	Yes
6 control vs 36 control	391.500	5.547	Yes
6 control vs 30 control	321.000	4.548	Yes
6 control vs 6 15 min	249.000	3.528	No
6 control vs 3015 min	232.500	3.294	Do Not Test
6 control vs 6 5 min	209.000	2.961	Do Not Test
6 5 min vs 30 5 min	346.000	4.902	Yes
6 5 min vs 36 5 min	346.000	4.902	Yes
6 5 min vs 3615 min	194.000	2.749	No
6 5 min vs 36 control	182.500	2.586	Do Not Test
6 5 min vs 30 control	112.000	1.587	Do Not Test
6 5 min vs 6 15 min	40.000	0.567	Do Not Test
6 5 min vs 30 15 min	23.500	0.333	Do Not Test
30 15 min vs 30 5 min	322.500	4.569	Yes
30 15 min vs 36 5 min	322.500	4.569	Yes
30 15 min vs 36 15 min	170.500	2.416	Do Not Test
30 15 min vs 36 control	159.000	2.253	Do Not Test
30 15 min vs 30 control	88.500	1.254	Do Not Test
30 15 min vs 615 min	16.500	0.234	Do Not Test
6 15 min vs 30 5 min	306.000	4.336	No

SHP-2 EDL

Film properties report SHP-2 EDL

Experimenter: Sreevani Uddemarri

Muscle : EDL Species: F344 X BN hybrid rat

Protein concentration: 60 µg/ml Gel type: 7.5% Tris-HCL SDSPAGE

Electrophoresis Voltage: 125V Transfer Voltage: 24V Duration: 45 min

Primary Antibody: SHP-2(Cell Signaling) Primary Antibody Dilution: 1:1000

Incubation Time: overnight @ 4°C Medium: 5% BSA

Secondary Antibody: Anti Rabbit Secondary Antibody Dilution: 1:1000

Incubation Time: 1hr @ room temp Medium: 5% milk in TBS-T

Exposure Time 3 min Molecular weight: 72 kDa

Lane 1: Rainbow Marker RPN756 3µl

Lane 2: 6 month control

Lane 3: 6 month 5 min

Lane 4: 6 month 15 min

Lane 5: 30 month control

Lane 6: 30 month 5 min

Lane 7: 30 month 15min

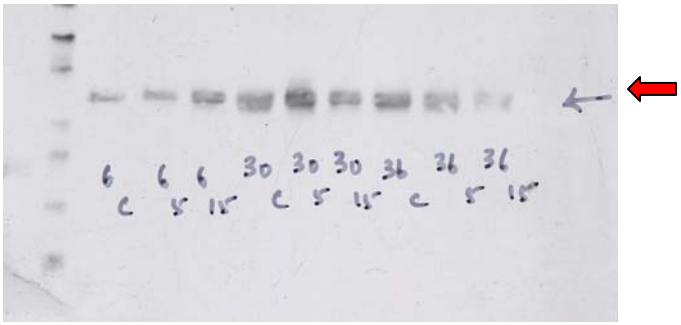
Lane 8: 36 month control

Lane 9: 36 month 5 min

Lane 10: 36 month 15 min

Lane 11: NIH 3T3 Activated Cell Extract 3µl

Lane 12: Biotinylated Ladder 3 µl



Raw data

This section represents the raw data tables Produced from spot densitometry of the immunoblot films.

SHP-2 in EDL Data set

Raw IOD values (relative percentage)

	6 month	30 month	36 month
Raw values	31	36.9	32.1
	31.2	38.1	30.7
	31.5	37.6	30.9
	17.3	40.4	42.3
	17.3	40.8	41.8
	16.1	41.1	42.9
	31.8	32.6	35.7
	31.2	31.7	37.2
	31.6	31.2	37.2
N	**9**	**9**	**9**
Mean	**26.55556**	**36.71111**	**36.75556**
Strandard Deviation	7.253811	3.948558	4.84874
Standard Error of the mean	**2.56461**	**1.396026**	**1.714288**
% Relative expression	**100**	**138.2427**	**138.41**
SE	**9.657526**	**5.257002**	**6.455479**

Statistics
One Way Analysis of Variance

Normality Test: Passed (P = 0.012)
Equal Variance Test: Passed (P = 0.486)

Group Name	N	Missing	Mean	Std Dev	SEM
6 control	9	0	26.556	7.254	2.418
30 control	9	0	36.711	3.949	1.316
36 control	9	0	36.756	4.849	1.616

Source of Variation	DF	SS	MS	F	P
Between Groups	2	621.532	310.766	10.165	<0.001
Residual	24	733.753	30.573		
Total	26	1355.285			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 0.967

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
36 mon vs. 6 mon	10.200	3	5.534	0.002	Yes
36 mon vs. 30 mon	0.0444	3	0.0241	1.000	No
30 mon vs. 6 mon	10.156	3	5.510	0.002	Yes

p-SHP-2 EDL

Film properties report p-SHP-2 EDL

Experimenter: Sreevani Uddemarri

Muscle : EDL Species: F344 X BN hybrid rat

Protein concentration: 60 µg/ml Gel type: 7.5% Tris-HCL SDSPAGE

Electrophoresis Voltage: 125V Transfer Voltage: 24V Duration: 45 min

Primary Antibody: p-SHP-2(Cell Signaling) Primary Antibody Dilution: 1:1000

Incubation Time: overnight @ 4°C Medium: 5% BSA

Secondary Antibody: Anti Rabbit Secondary Antibody Dilution: 1:1000

Incubation Time: 1hr @ room temp Medium: 5% milk in TBS-T

Exposure Time 3 min Molecular weight: 72 kDa

Lane 1: Rainbow Marker RPN756 3µl

Lane 2: 6 month control

Lane 3: 6 month 5 min

Lane 4: 6 month 15 min

Lane 5: 30 month control

Lane 6: 30 month 5 min

Lane 7: 30 month 15min

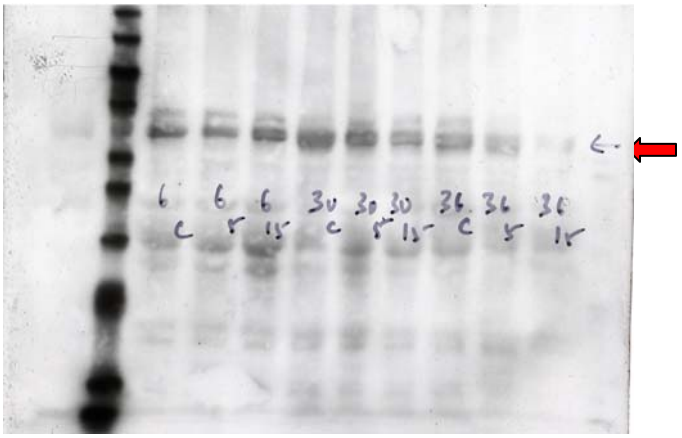
Lane 8: 36 month control

Lane 9: 36 month 5 min

Lane 10: 36 month 15 min

Lane 11: NIH 3T3 Activated Cell Extract 3µl

Lane 12: Biotinylated Ladder 3 µl



Raw data

This section represents the raw data tables Produced from spot densitometry of the immunoblot films.

p-SHP-2 in EDL Data set

	6 Control	**6 5 min**	**6 15 min**	**30 Control**	**30 5 min**	**30 15 min**	**36 Control**	**36 5 min**	**36 15 min**
Raw values	15.7	11	13.8	16.4	14.3	9.8	10.4	6.6	2.2
	14.5	9.9	13	16.9	15.5	10.2	10.8	6.7	2.4
	15	9.4	12.9	16.6	15.7	9.9	10.9	7	2.5
	11.6	11.6	12.4	11.2	11.5	10.1	8.9	11.2	11.3
	9.9	10.7	13	9.9	11.3	10.4	10.8	12.4	11.6
	11.5	11.9	12.8	9.2	10.6	10.1	10.2	12.4	11.3
N	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**13.03333**	**10.75**	**12.98333**	**13.36667**	**13.15**	**10.08333**	**10.33333**	**9.383333**	**6.883333**
Strandard Deviation	2.338946	0.964883	0.457894	3.639047	2.280132	0.213698	0.752773	2.9027	4.949916
Standard Error of the mean	**1.046008**	**0.431509**	**0.204776**	**1.627432**	**1.019706**	**0.095568**	**0.33665**	**1.298127**	**2.21367**
Relative Expression Level	**100**	**82.4808**	**99.6164**	**102.5575**	**100.8951**	**77.3657**	**79.2839**	**71.9949**	**52.8133**
Standard error of the mean	**8.0256**	**3.3108**	**1.5712**	**12.4867**	**7.8238**	**00.7333**	**2.583**	**9.9601**	**16.9847**

Raw IOD values (relative percentage)

Statistics

One Way Analysis of Variance

Normality Test: Passed (P = 0.151)
Equal Variance Test: Failed (P = <0.001)
Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
6 control	6	0	13.050	11.500	15.000
6 5 min	6	0	10.850	9.900	11.600
6 15 min	6	0	12.950	12.800	13.000
30 control	6	0	13.800	9.900	16.600
30 15 min	6	0	10.100	9.900	10.200
30 5 min	6	0	12.900	11.300	15.500
36 control	6	0	10.600	10.200	10.800
36 5 min	6	0	9.100	6.700	12.400
36 15 min	6	0	6.900	2.400	11.300

H = 21.588 with 8 degrees of freedom. (P = 0.006)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.006)

T

SHP-2 Soleus

Film properties report SHP-2 Soleus

Experimenter: Sreevani Uddemarri

Muscle : Soleus Species: F344 X BN hybrid rat

Protein concentration: 60 µg/ml Gel type: 7.5% Tris-HCL SDSPAGE

Electrophoresis Voltage: 125V Transfer Voltage: 24V Duration: 45 min

Primary Antibody: SHP-2 (Cell Signaling) Primary Antibody Dilution: 1:1000

Incubation Time: overnight @ 4°C Medium: 5% BSA

Secondary Antibody: Anti Rabbit Secondary Antibody Dilution: 1:1000

Incubation Time: 1hr @ room temp Medium: 5% milk in TBS-T

Exposure Time 3 min Molecular weight: 72kDa

Lane 1: Rainbow Marker RPN756 3µl

Lane 2: 6 month control

Lane 3: 6 month 5 min

Lane 4: 6 month 15 min

Lane 5: 30 month control

Lane 6: 30 month 5 min

Lane 7: 30 month 15min

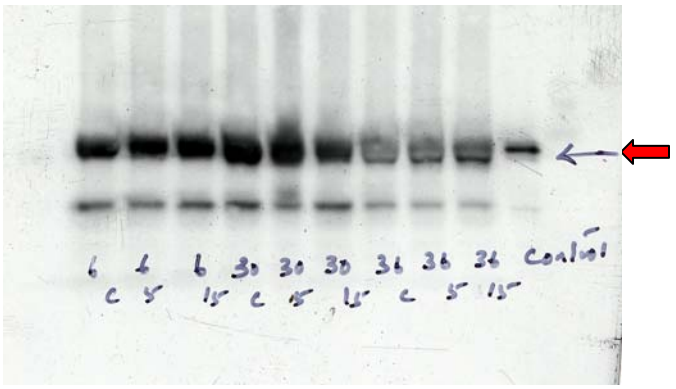
Lane 8: 36 month control

Lane 9: 36 month 5 min

Lane 10: 36 month 15 min

Lane 11: NIH 3T3 Activated Cell Extract 3µl

Lane 12: Biotinylated Ladder 3 µl



Raw data

This section represents the raw data tables Produced from spot densitometry of the immunoblot films.

SHP-2 in Soleus Data set

Raw IOD values (relative percentage)

	6 month	30 month	36 month
Raw values	13.8	14	6.3
	13.6	13.3	6.3
	13.4	13.6	7.1
	12.9	13.2	8.1
	12.4	14.2	6.6
	11.6	13.5	8.8
N	6	6	6
Mean	12.95	13.63333	7.2
Standard Deviation	0.833667	0.393277	1.035374
Standard Error of the mean	0.372827	0.175879	0.463033
% Relative expression	100	105.2767	55.5985
SE	2.879	1.3581	3.5755

Statistics
One Way Analysis of Variance

Normality Test: Passed (P > 0.200)
Equal Variance Test: Passed (P = 0.235)

Group Name	N	Missing	Mean	Std Dev	SEM
6 control	6	0	12.950	0.834	0.340
30control	6	0	13.633	0.393	0.161
36control	6	0	7.200	1.035	0.423

Source of Variation	DF	SS	MS	F	P
Between Groups	2	149.834	74.917	116.957	<0.001
Residual	15	9.608	0.641		
Total	17	159.443			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Means	p	q	P	P<0.050
30control vs. 36control	6.433	3	19.689	<0.001	Yes
30control vs. 6control	0.683	3	2.091	0.328	No
6control vs. 36control	5.750	3	17.598	<0.001	Yes

p-SHP-2 Soleus

Film properties report p-SHP-2 Soleus

Experimenter: Sreevani Uddemarri

Muscle : Soleus Species: F344 X BN hybrid rat

Protein concentration: 60 µg/ml Gel type: 7.5% Tris-HCL SDSPAGE

Electrophoresis Voltage: 125V Transfer Voltage: 24V Duration: 45 min

Primary Antibody: p-SHP-2(Cell Signaling) Primary Antibody Dilution: 1:1000

Incubation Time: overnight @ 4°C Medium: 5% BSA

Secondary Antibody: Anti Rabbit Secondary Antibody Dilution: 1:1000

Incubation Time: 1hr @ room temp Medium: 5% milk in TBS-T

Exposure Time 3 min Molecular weight: 72 kDa

Lane 1: Rainbow Marker RPN756 3µl

Lane 2: 6 month control

Lane 3: 6 month 5 min

Lane 4: 6 month 15 min

Lane 5: 30 month control

Lane 6: 30 month 5 min

Lane 7: 30 month 15min

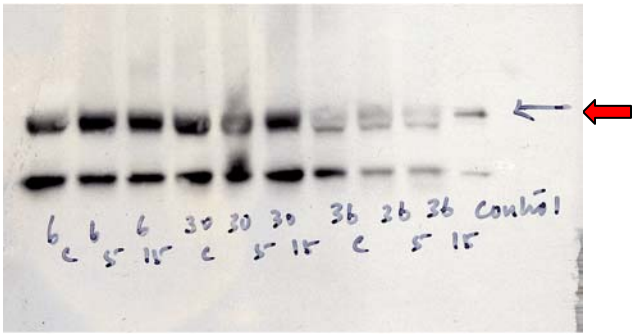
Lane 8: 36 month control

Lane 9: 36 month 5 min

Lane 10: 36 month 15 min

Lane 11: NIH 3T3 Activated Cell Extract 3µl

Lane 12: Biotinylated Ladder 3 µl



Raw data

This section represents the raw data tables Produced from spot densitometry of the immunoblot films.

p-SHP-2 in Soleus Data set

Raw %IOD values

Raw IOD values (relative percentage)

	6 Control	**6 5 min**	**6 15 min**	**30 Control**	**30 5 min**	**30 15 min**	**36 Control**	**36 5 min**	**36 15 min**
Raw values	13.4	17.1	14.5	15	11.4	14.7	5	5.8	3.2
	13.8	17.7	15.7	16.1	11	13.9	4.4	4.6	2.9
	14.2	16.8	15.3	14.9	11.5	14.5	4.5	5.3	2.9
	9	10.5	11.3	16.5	16.3	20	5.7	5.7	4.9
	9.8	10.2	11.8	16.8	14.7	18.8	4.6	7	6.3
	8.9	11.2	12.4	16.7	15.9	17.9	3.9	6.6	6.4
N	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**11.51667**	**13.91667**	**13.5**	**16**	**13.46667**	**16.63333**	**4.683333**	**5.833333**	**4.433333**
Strandard Deviation	2.533311	3.622936	1.89842	0.848528	2.436938	2.584312	0.611283	0.868716	1.660923
Standard Error of the mean	**1.132931**	**1.620226**	**0.848999**	**0.379473**	**1.089832**	**1.155739**	**0.273374**	**0.388501**	**0.742788**
Relative Expression Level	**100**	**120.8394**	**117.2214**	**138.9291**	**116.932**	**144.4284**	**40.6657**	**50.6512**	**38.4949**
Standard error of the mean	**9.8373**	**14.0685**	**7.3719**	**3.295**	**9.4631**	**10.0354**	**2.3737**	**3.3734**	**6.4497**

Statistics
One Way Analysis of Variance

Normality Test: Passed (P > 0.200)
Equal Variance Test: Failed (P = <0.001)
Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
6 control	6	0	11.600	9.000	13.800
6 5 min	6	0	14.000	10.500	17.100
6 15 min	6	0	13.450	11.800	15.300
30 control	6	0	16.300	15.000	16.700
30 5 min	6	0	13.100	11.400	15.900
30 15 min	6	0	16.300	14.500	18.800
36 control	6	0	4.550	4.400	5.000
36 5 min	6	0	5.750	5.300	6.600
36 15 min	6	0	4.050	2.900	6.300

H = 41.601 with 8 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0.05
3015 min vs 36 control	220.000	5.709	Yes
3015 min vs 3615 min	219.000	5.683	Yes
3015 min vs 365 min	185.000	4.801	Yes
3015 min vs 6control	108.000	2.803	No
3015 min vs 615 min	61.500	1.596	Do Not Test
3015 min vs 305 min	60.500	1.570	Do Not Test
3015 min vs 65 min	45.500	1.181	Do Not Test
3015 min vs 30control	0.500	0.0130	Do Not Test
30 control vs 36control	219.500	5.696	Yes
30 control vs 3615 min	218.500	5.670	Yes
30 control vs 365 min	184.500	4.788	Yes
30 control vs 6control	107.500	2.790	Do Not Test
30 control vs 615 min	61.000	1.583	Do Not Test
30control vs 30 5 min	60.000	1.557	Do Not Test
30control vs 6 5 min	45.000	1.168	Do Not Test
65 min vs 36 control	174.500	4.528	Yes
65 min vs 3615 min	173.500	4.502	Yes
65 min vs 365 min	139.500	3.620	No
65 min vs 6control	62.500	1.622	Do Not Test
65 min vs 615 min	16.000	0.415	Do Not Test

BUFFERS AND PROTOCOLS

Krebs-Ringers Solution

Chemicals

All chemicals listed below were purchased from Sigma Chemical Company

NaCl	KCl
$MgSO_4$	NaH_2PO_4
$CaCl_2$	$NaHCO_3$
α-D-Glucose	d-tubocurarine chloride

Preparation of Concentrated (10x) Krebs-Ringers stock solution

10x Salt Stock (in 1000ml deionized water)

68.96 g NaCl	3.5 g KCl
14 g $MgSO_4$	1.5 g NaH_2PO_4

10x Calcium Stock (in 1000ml deionized water)

2.78 g $CaCl_2$

10x Bicarbonate Stock (in 1000ml deionized water)

21.0 g $NaHCO_3$

**Stock solutions stored in refrigerator*

Final Concentrations of Krebs-Ringers Working Solution

118 mM NaCl	4.7 mM KCl
1.2 mM $MgSO_4$	1.2 mM NaH_2PO_4
2.5 mM $CaCl_2$	25mM $NaHCO_3$

10 mM α-D-Glucose

Preparation of Krebs-Ringers working solution

Working solution is prepared daily and discarded at the end of the experiment. A working KRS is prepared by adding 100 ml of 10 salt stock + 200 ml deionized water. Then, add 100 ml of 10x calcium stock + 200 ml deionized water. Finally, add 100 ml of 10 x bicarbonate stock. Add 1.8 grams of α-D-Glucose (optional add 0.017 grams d-tubocurarine chloride) and fill with deionized water to ≤1000 ml total volume.

* Oxygenate Ringer's solution for 10 minutes with 5% Co_2 and 95% O_2

* Adjust pH to 7.4 using 1N HCl or 1N NaOH.

* Fill to 1000 ml total volume.

T-PER lysis buffer for tissue pulverization

Makes 5 ml TPER lysis buffer.

1. 0.5 M EDTA	10 µl
2. 0.1 M EGTA (MW 468.4):	50 µl
3. 1.0 M MgCL2:	7.5 µl
3. 0.1 M NaVO3:	50 µl
5. 0.5 M PMSF:	1 µl
6. Protease Inhibitor Cocktail:	1 µl

Procedure:

1. Pre weight sample vial
2. Pulverized sample using liquid nitrogen
    1. Place large mortis on ice,
    2. Place small mortis inside large mortis
    3. Fill small mortis with liquid nitrogen
    4. Place frozen sample in liquid nitrogen filled small mortis
    5. Dip porcelain pestle in liquid nitrogen (to prevent sample from melting and freezing to pestle.
    6. Immediately grind sample with porcelain pestle Do not allow sample to warm
    7. Repeat steps c, and e-f frequently until sample has formed a powder.
    8. Take precaution to minimize exposure of skin to liquid nitrogen.
3. Transfer pulverized sample to pre-weighed sample vial
4. Weigh sample with sample vial

5. Add 1000µl lysis buffer per 1 g of pulverized sample and vortex
6. Incubate on ice for 30 minutes, vortex every 5 minutes.
7. Centrifuge at 14,000rpm at 4°C for 5 minutes
8. Transfer supernatant to fresh tubes and store at -80°C
9. Perform Bradford's protein assay (Sample may be concentrated using a speed-vac if required)
10. Dilute with appropriate amount of water to obtain 25 or 50µl of 5µg/µl solution. (Storage -80°C)
11. Add equal volume of Laemelli's Sample buffer and store at -20°C.

Xylazine – Ketamine anesthesia

Recipe #1

1. In a sterile 10 ml bottle with a rubber stopper, mix 8.75 ml of Ketamine (100 mg/ml), and 1.25 ml of Xylazine (100 mg/ml). Shake well before use.
2. Identify the bottle, writing: "Xylazine – Ketamine for rat: 0.05 – 0.10 ml/100 g IP", and the date the mix was prepared and your initials.
3. Write the expiration date on the bottle, selecting the earliest expiration date between ketamine, xylazine, or water, for a maximum of 3 months. Keep away from light, in a cool place.
4. Administer 0.05 – 0.10 ml/100 g IP.
5. Repeat as required with 1/3 to 1/2 dose at a time (approximately every 30 minutes)
6. Prevent heat loss until the animal recovers.

Recipe #2

1. In a sterile 10 ml bottle with a rubber stopper, mix 3.75 ml of Ketamine (100 mg/ml), and 0.5 ml of Xylazine (100 mg/ml). Shake well before use.
2. Identify the bottle, writing: "Xylazine – Ketamine for rat: 0.2 ml/100 g IP", and the date the mix was prepared and your initials.
3. Write the expiration date on the bottle, selecting the earliest expiration date between ketamine, xylazine, or water, for a maximum of 3 months. Keep away from light, in a cool place.
4. Administer 0.2 ml/100 g IP.

5. Repeat as required with 1/3 to 1/2 dose at a time (approximately every 30 minutes)
6. Prevent heat loss until the animal recovers.

References

[Flecknell, 1996 #161][DF, 1997 #162]

5% Milk in TBS-T

Milk (powdered Kroger nonfat dry milk)	12.5 g
TBS-T	250 ml

3% Milk in TBS-T

Milk (powdered Kroger nonfat dry milk)	7.5 g
TBS-T	250 ml

Transfer Buffer

Tris Base	3.03 g
Glycine	14.4 g

Bring to 800 ml with diH_2O

Bring to 1000ml with pure methanol(100%).

5% BSA in TBS-T

BSA	12.5 g
TBS-T	250 ml

Bradford's protein assay

The Branford assay is the standard means of measuring the concentration of protein within a volume of supernatant.

1. Label curvet for each BSA protein standard 0.25, 0.5, 1 ,1.5 and 2
2. Label three curvets for each sample to be assayed.
3. Load 20 µl of each BSA protein standard into the appropriately labeled curvet.
4. Load 16 µl of water into each pre-labeled sample curvet.
5. Load 4 µl of each sample of unknown protein concentration into the appropriately labeled curvet containing the previously loaded 16 µl of water.
6. Load 1000 µl of Comassie stain into all curvets and vortex
7. Using the mass spectrometer with the wavelength set at 595 nm, select the standard curve setting.
8. Load BSA standard loaded curvets into the spectrometer, enter standards concentration, and run unit to set standard curve (record the r^2 value).
9. Remove all curvets with standards accept the blank in the first cell.
10. Place curvets with unknowns and select test unknown on spectrometer.
11. Run appropriate program and record data for each sample.
12. Load data into excel spread sheet and multiple the concentration by 5 to determine unknown concentrations
13. If concentration are too low, Speed vac to increase concentration of protein in sample, and repeat assay.

14. Calculate the appropriate volume of diH_2O needed to covert all sample to the same protein concentrations.
15. Dilute with appropriate amount of water to obtain 25 or 50µl of 6µg/µl solution. (Storage -80°C)
16. Add equal volume of Laemelli's Sample buffer and store at -80°C

CURRICULUM VITAE

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Objective
To obtain a MS in Biology

Professional Highlights

MBBS (Bachelor of Medicine and Bachelor of Surgery)

Summary of Research Skills

Rat surgeries and sample processing

Immunoprecipitation and silver staining

Western blotting

Understanding of SAS (Software Analysis Program) and Sigma Stat

Alpha Ease and Densitometries

Cell culture

Work History

Practiced medicine for a year (April 1997-August 1998) in a community hospital in India

Education

Medical school -Rangaraya Medical College, Kakinada, India

Internship-Osmania Medical College, Hyderabad, India

Passed Medical licensing exams in United States

Future Goal

Do fellowship in Endocrinology and be a Research Physician

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